(54) Title: DIFFERENTIAL BINDING AFFINITIES AND DISSOCIATION ASSAYS BASED THEREON

(57) Abstract

A method for assaying for the presence of analyte in a sample based on differential binding affinity involves detecting dissociation of a complex of receptor and ligand in the presence of analyte. The receptor binds the analyte with high affinity and with the ligand with low affinity. The receptor-ligand complex may be formed in situ or may be preformed. In the presence of free analyte, the receptor releases from the receptor-ligand complex and binds free analyte. Release of the receptor-ligand complex is detectable. A kit for performing release assays to detect the presence of analyte is also provided.

DIFFERENTIAL BINDING AFFINITIES
AND DISSOCIATION ASSAYS BASED THEREON

1. FIELD OF THE INVENTION

5   The present invention relates to methods
for determining the presence of an analyte in a
sample. More particularly, homogeneous liquid-phase
and heterogeneous liquid-phase/solid-phase release
assays that are highly specific and sensitive are
10 provided.

2. BACKGROUND OF THE INVENTION

        Immunoassays utilize the specific binding
capabilities of antibodies to detect the presence of
15 target molecules in solution. Although the general
principle is applicable to a broad range of problems,
major commercial interest has centered on medical
diagnostic applications for a wide variety of analytes
in biological fluids such as blood, saliva, and urine.
20   Several types of immunoassays, useful for
distinct applications, already exist. Each such assay
type requires a way of distinguishing whether binding
sites on an antibody are occupied or free. Typically
this is accomplished by means of label such as an
25 atom, molecule, enzyme or particle attached
permanently to either the antibody or to an analog of
the analyte.
        Sensitivity and specificity are key
parameters of an immunoassay. Specificity relates
30 primarily to the antigen binding site of the antibody,
which is inherent to selection of variable region gene
segments and is independent of the assay
configuration. Sensitivity relates primarily to the
affinity of the antibody for its ligand(s) and to the
35 inherent detectability of the label. For example,

radioisotopes, used for radioimmunoassay, can be
detected at significantly lower concentrations than
fluorescent molecules. Enzyme labels are detectable
at concentrations similar to fluorescent labels. When
5 substrates that produce fluorescent or
chemiluminescent products are used with enzyme labels,
the sensitivity of resulting immunoassays is
comparable or greater than with radioisotope labels.

Many conventional assay techniques are
10 considered competitive in that the analyte and labeled
component have comparable affinities for the antibody
binding site. One example of such a competitive
method is found in U.S. Patent 3,817,837 by Rubenstein
and Ullman which describes a technique in which ligand
15 and enzyme-bound-ligand compete for antibody binding
sites. Since binding of the antibody to the enzyme-
bound-ligand alters its enzymatic activity, the
concentration of ligand present can be estimated by
measuring the rate at which such a mixture converts
20 substrate to product.

Immunoassays can be further characterized as
homogeneous and heterogeneous. In a heterogeneous
method, the label is equally detectable in bound and
unbound states. To obtain any meaningful assay
25 results physical separation of the bound versus
unbound antibody is required. A common strategy for
accomplishing this separation entails associating the
label to a solid phase which can be physically
separated from the liquid phase prior to the detection
30 step. A typical heterogeneous assay is the Tandem EIA
from Hybritech, Inc.

In a homogeneous method, the detectable
property of the label is inherently different
depending on whether bound or unbound. In its bound
35 state, the label will haver greater or lesser signal

intensity. Usually, binding of antibody to the
labeled ligand causes a decrease in signal intensity,
e.g., when the label is an enzyme. Typical products
in this category include the EMIT line of enzyme

5 immunoassays from Syva Company and the TDX line of
fluorescence polarization immunoassays from Abbott
Diagnostics.

Two further characteristics of immunoassays
are particularly noteworthy. These are the minimal

10 concentration of analyte that can be detected, and the
dynamic range of detection. The dynamic range is the
range of analyte concentrations over which signal from
a label changes from zero to maximum. The order in
which the sample, the antibody, and a labeled

15 component are combined can significantly affect both
of these key parameters by affecting the degree of
binding of the labeled component, which in turn
affects detection of the label.

In certain known assay methods, the antibody

20 and the analyte are combined prior to addition of the
labeled component. In others, the analyte and labeled
component are combined prior to addition of the
antibody. Each of these cases requires providing two
separate reagents that are combined with the sample

25 containing the analyte. The need for two such
separate reagents can be inconvenient and result in a
more cumbersome, complex method. Moreover, because
precise volumetric measurement of each reagent is
critical to good assay performance, the necessity of

30 two measuring steps can cause errors which may lead to
distorted results.

One method to improve assay precision and
thereby enhance assay sensitivity is to provide a
premixed complex of the antibody and labeled

35 component. This is problematic, however, because the

binding reaction is generally found to be
irreversible. Thus, when a complex of the labeled
analyte and antibody are combined with a solution
containing the analyte, no appreciable displacement of
5 bound label occurs in a meaningful time frame (seconds
to minutes).

The present invention relates to assay
methodology that employs a complex of receptor and a
ligand, wherein the receptor-ligand complex
10 dissociates in the presence of an analyte and a stable
receptor-analyte complex is formed. Dissociation of
the receptor-ligand complex in the presence of analyte
is detectable thereby positively indicating the
presence of analyte in a test sample. Methods for
15 designing, preparing, using, and stabilizing such
complexes are taught. The methodology is applicable
both to homogeneous assays and heterogeneous assays
for analytes encompassing a broad range of types and
sizes.

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3. SUMMARY OF THE INVENTION

The present invention provides for
heterogeneous and homogeneous release assay methods
for detecting the presence of an analyte in a sample.
25 A kit for performing the assay method of the invention
is also provided.

According to the present method, a test
sample is contacted with a receptor-ligand complex.
The receptor has much a higher association constant
30 for analyte than ligand. When the receptor-ligand
complex dissociates into its receptor and ligand
components in the presence of free analyte in the test
sample, receptor binds the free analyte to form a
stable receptor-analyte complex, which is
35 substantially unaffected by the presence of free

ligand. In accordance with the present method,
dissociation of the receptor-ligand complex, i.e.,
release of the receptor and ligand, is a detectable
event indicating the presence of analyte in the test
5   sample. Detection of either dissociated ligand or
receptor is contemplated. Means for detection are
detailed _infra_ in sections 5.2, 5.2.1 and 5.2.2.

       In one embodiment of the invention a kit for
carrying out the present method is provided. The kit
10 includes either preformed receptor-ligand complex or
receptor and ligand which are mixed to form the
complex prior to conducting the assay. As can be
appreciated, when the pre-formed complex is provided,
apart from test sample, only this single reagent may
15 be required to carry out the assay.

       As shown in the Examples, _infra_, the present
invention provides surprisingly greater sensitivity,
specificity, accuracy and range of detection than
conventional association assays or competitive
20 dissociation assays.

3.1.   DEFINITIONS

       Analyte -- molecule of interest in an assay.
       Ligand -- molecule capable of binding to a
25 receptor specific for analyte with substantially lower
affinity than that of analyte binding to the receptor.
       Receptor -- molecule capable of specifically
binding to analyte or ligand. In each case, a stable
complex is formed, but the association constant of
30 receptor for analyte is higher than that for ligand.
Dissociation of the receptor-ligand complex into its
receptor and ligand components in the presence of free
analyte results in release of the receptor which binds
to free analyte, thereby forming a stable receptor-

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analyte complex substantially unaffected by the
presence of free ligand.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Release of anti-cotinine antibody
in the presence of cotinine in a urine matrix sample.
Anti-cotinine was labeled with peroxidase and
complexed with solid phase cotinine ligand. Free
cotinine was added and released anti-cotinine was
detected by measuring enzyme activity in the
supernatant after 2 min incubation. Ligands were
cotinine conjugated to BGG, using the linker
aminocaproic acid (open squares) or p-aminobenzoic
acid (solid squares).

Figure 2. Release of anti-cotinine antibody
after a 10 minute incubation in a complex with
immobilized ligand. A synthetic urine matrix sample
was spiked with cotinine. Ligands are the same as for
Figure 1.

Figure 3. Release of anti-cotinine antibody
after a 2 minute incubation in urine and synthetic
urine matrix samples. Release of labeled anti-
cotinine was detected from cotinine-aminocaproyl-BGG
in urine (solid squares) and synthetic urine (open
squares) and from cotinine-benzoyl-BGG in urine
(asterisks) and synthetic urine (solid triangles).

Figure 4. Deactivation and inhibition of a
glucose-6-phosphate dehydrogenase-hydroxycotinine
conjugate. Normal enzyme activity (solid squares),
conjugate activity (open squares), and conjugate
activity in the presence of antibody (asterisks) are
shown.

Figure 5. Inhibition and release of a
hydroxycotinine-glucose-6-phosphate dehydrogenase
conjugate. The enzyme activity (A_{340} vs time) of enzyme

conjugate (solid squares), enzyme conjugate plus antibody to cotinine (solid triangles), and enzyme conjugate + antibody + free cotinine (open squares) are shown.

5   Figure 6. Automated release assay for cotinine. Release in the presence of cotinine was measured on an automated analyzer after <1 (dots), 18 (crosses), and 22 (asterisks) hours of complex incubation.

10   Figure 7. Efficiency of various low affinity analogs of cotinine conjugated to glucose-6-phosphate dehydrogenase. Conjugates of trans-hydroxy cotinine (extensively conjugated (dots), or partially conjugated (vertical lines, asterisks)), cis-

15 hydroxycotinine (crosses) and carboxycotinine (open squares) to glucose-6-phosphate dehydrogenase were tested as release ligands in a solid phase assay.

    Figure 8. Structure of (a.) nicotine, (b.) cotinine, (c.) N-isopropyl-4-carboxy-norcotinine and

20 (d.) N-propyl-4-carboxy-norcotinine.

    Figure 9. ELISA format release assay. Microtiter plates were coated with cis hydroxycotinine G-6-PDH (asterisks); N-isopropyl-norcotinine G-6-PDH (plus signs); N-propyl-norcotinine G-6-PDH [open

25 stippled squares].

    Figure 10. Comparison of the dose response curves of an associative homogenous assay for cotinine (NiMA) with the release assay. Associative NiMA assay (plus signs); release assay (open stippled square).

30   Figure 11. Standard curve for the release assay of cotinine by the homogeneous method.

    Figure 12. Benzoylecgonine release assay using a microtiter plate format. Matrix effects were observed depending on the liquid medium, which was

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either water (solid squares) or urine (crosses). BE
is benzoylecgonic.

Figure 13. Effect of incubation time on
benzoylecgonine release assay using a microtiter plate
5   format. Supernatants were obtained 0 (solid squares),
2 (open squares) and 10 (asterisks) minutes after
addition of free benzoylecgonine (BE) and checked for
release of labeled anti-benzoylecgonine.

Figure 14. Low affinity chromatography of
10 anti-beta hCG. Rabbit antiserum to human chorionic
gonadotropin (hCG) was applied to a sheep gonadotropin
affinity column and eluted with 13 ml PBS, 20 ml of pH
4 buffer, and 10 ml of pH 8 buffer. Protein (light
cross-hatch); anti-hCG (dark cross-hatch); IgG (double
15 cross-hatch).

5.   DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention a
method for detecting the presence of analyte in a
20 sample and kits therefore are provided. A test sample
can be any body fluid, such as urine, blood, serum,
saliva, bodily exudate, etc., suspected of containing
the target analyte. The release assay method of the
invention involves contacting a test sample with a
25 receptor-ligand complex and, if analyte is present,
detecting dissociation of that complex into its
receptor and ligand components. Dissociation of the
complex in the presence of analyte results in release
of receptor and ligand. Upon release, receptor binds
30 with free analyte, forming a receptor-analyte complex
that remains stable in the presence of free ligand.
Either dissociated ligand or receptor can be detected
to indicate the presence of analyte in the sample.
The assay method of the invention may be performed in
35 either homogeneous or heterogeneous formats. Specific

details of each format are pr vided _infra_ in Section
5.2.

A release assay of the present invention
allows preparation of a complex in which receptor
5   binding sites and ligand are present in approximately
equal concentration, i.e., quantitative complex
formation, although this is not essential. Having
each element present in equal concentration enhances
sensitivity, since when receptor is present in excess,
10 it can bind analyte without releasing from receptor-
ligand complex.

In order to achieve a receptor-ligand
complex in which the number of binding sites of each
element are present in substantially equimolar
15 amounts, the receptor and ligand are incubated for at
least ten seconds prior to exposure to sample.
Preferably the incubation time is greater than about
one hour. A long incubation time allows formation of
the most stable complexes during repeated release and
20 binding reactions when one element, either receptor or
ligand, is present in excess. After the low affinity
binding reaction reaches equilibrium, the receptor-
ligand complex is isolated from the excess element.
Isolation of the complex may be either by size
25 exclusion chromatography, density gradient
centrifugation, low affinity chromatography, or other
techniques for separating complexes from their
components. Alternatively, the receptor and ligand
may be mixed in equal binding site concentrations and
30 incubated to allow substantially quantitative binding
to create stable complexes. Formation of the reagent-
ligand complex is discussed more fully in Section
5.1., _infra_.

The kit of the present invention preferably
35 includes preformed receptor-ligand complex, thereby

eliminating the need to prepare reagents prior to
conducting an assay. However, providing both receptor
and ligand separately, and mixing them prior to the
assay, is within the scope of the invention.

5   The present invention is based on the
principle that dissociation of a complex of receptor
and ligand in the presence of analyte will result in
association of receptor and analyte rather than
reassociation of receptor and ligand when the

10   association constant of receptor and analyte is
greater than the association constant of receptor and
ligand. The dissociation of a receptor-ligand
complex, release of receptor and ligand, and binding
of receptor to analyte to form a stable complex is

15   termed herein the release reaction. The corollary of
a release reaction is that free ligand will not affect
the stability of the receptor-analyte complex, so
that the receptor will not bind to free ligand, i.e.,
the release reaction is not reversible. Similarly a

20   stable receptor-ligand complex will not dissociate and
release receptor to bind a second low affinity cross
reactive ligand because there is no kinetic or the
thermodynamic gain. The dissociation constant of even
a low affinity receptor-ligand complex is relatively

25   low, and binding to another ligand with an equivalent
affinity constant does not result in a free energy
change. This results in a sensitive, highly specific,
highly accurate (minimally cross reactive) assay.

However, when analyte binds receptor with

30   high affinity and the association constant is high,
dissociation of the receptor from the receptor ligand
complex and binding to the analyte will occur readily.
The high association rate will result in fast "pick-
up" of dissociated receptor. The release reaction is

35   thermodynamically favorable, since a higher affinity

constant will give a n gative net free energy change.
The thermodynamics and kinetics will drive a release
reaction in the presence of analyte, and will result
in no change in the absence of analyte.

5   Preferably the affinity constant of binding
of receptor to ligand will be about 10%, and more
preferably about 1%, of the affinity constant of
binding of receptor to analyte. This can be observed
qualitatively as relative binding, e.g., by apparent
10 activity in an assay.

Because the thermodynamics and kinetics of
the release reaction favor binding of receptor to
analyte over binding of receptor to ligand,
macroscopically analyte appears to induce the release
15 reaction, i.e., dissociation of the receptor-ligand
complex.

Since the release reaction depends on a high
affinity association of receptor and analyte, it is
sensitive and specific. That is, receptor will bind
20 low concentrations of analyte. Dependence of the
release reaction on the differential affinity binding
further increases specificity. Receptor will not
dissociate and bind cross-reactive analogs of the
analyte unless the binding constant is much higher
25 than the binding constant of receptor and ligand.

It is important to emphasize that a
significant portion of the complex must dissociate, or
the background "noise" in the system will be too
great. For example, if only 1% of the receptor-ligand
30 complex is dissociated, 99% of the system is
unaffected. If the standard deviation of measurement
is 1% (equivalent to 99 ± 1%), which represents an
excellent coefficient of variation in immunoassay
systems, then the effect of 1% release would be 1% ±
35 1%, nullifying any significance. By taking advantage

of the thermodynamic and kinetic impetus of the release reacti n, the present assay provides for significant dissociation of receptor-ligand complex, i.e., release above the baseline levels. Moreover,

5   the release assay format provide for a broad concentration range over which analytes can be detected.

The receptor-ligand complex need not be fully dissociated for effectiveness, however. For

10 example, when a solid phase preparation of hydroxycotinine linked to a carrier protein (e.g., glucose-6-phosphate dehydrogenase) is reacted with sample, only 5-10% of total antibody labeled with the enzyme horseradish peroxidase is displaced by analyte.

15 The exact percentage is difficult to ascertain since enzyme activity on a solid phase is known to be less than that of enzyme in liquid phase. Nevertheless, the amount of release (greater than 5%) provides a significant signal over background. In a homogeneous

20 release assay, the sample containing cotinine induces 100% reversal of enzyme inhibition, which indicates 100% release.

5.1. THE RECEPTOR-LIGAND COMPLEX

25   An effective release assay requires formation of a stable low affinity receptor-ligand complex at equilibrium. This complex usually forms more slowly than conventional complexes, e.g., receptor-analyte, or antibody-antigen complexes, etc.

30 Dissociation occurs readily during initial receptor-ligand complex formation. However, after a suitable incubation period, the receptor-ligand complex becomes stable. The stability of the complex is in part a function of the design of the ligand, as well as

35 incubation time of ligand and receptor. The

appropriate incubation time is readily determined for
each receptor and ligand combination. Generally,
however, receptor and ligand should be incubated at
least 10 seconds, preferably at least 10 minutes, and
5   more preferably longer than 1 hour, prior to exposure
to analyte. In cases where the receptor-ligand
complex is isolated prior to performing the assay,
both receptor and ligand binding sites are present in
substantially equimolar amounts and the incubation
10   time is not important (see Section 5, supra). Under
appropriate conditions, e.g., the presence of salts
such as sodium chloride, or stabilizing agents such as
glucose, or both, the stable receptor-ligand complex
will remain releasable over a long time period --
15   days, weeks or longer. According to the present
invention, a stable receptor-ligand complex may be in
solution, or it may be dried. In an Example, infra, a
release assay could be run six days after antibody-
ligand complex formation in the presence of 5% sodium
20   chloride. In another Example, infra, the presence of
glucose stabilized a dry antibody-ligand complex.

Although the present invention is not bound
by any theory or hypothesis, it is believed that
formation of a stable receptor-ligand complex supports
25   a model of molecular accommodation between the ligand
and the receptor. The equilibrium of this binding
favors a configuration of the complex which stabilizes
it, meaning that the effective affinity of the complex
may, and probably must be, higher in the mature
30   complex than initially. Only by creating a stable
complex can one assure that dissociation is specific,
and is driven by the much higher affinity of the
receptor for the analyte.

Unless a release type assay is formatted
35   with differential binding affinities, and preferably

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includes the use of stabilizing agents, undesirable
irreversible receptor-ligand complex formation can
occur. For example, in a conventional homogeneous
immunoassay, if the complex between antibody and
5   ligand labeled with enzyme is allowed to remain in
solution overnight, the enzyme is gradually denatured.
Evidently molecular accommodation of the antibody-
ligand complex generally occurs in such a way as to
denature the enzyme rather than producing only
10   reversible inhibition.

The present invention overcomes these
limitations. Continuing with the example of an enzyme
label, in the release system, incubating a complex in
the presence of high salt to prevent formation of an
15   unreleaseable receptor-ligand complex, in which ligand
or receptor is labeled with enzyme, preserves the
enzyme activity as well as the ability to dissociate
the receptor-ligand complex. When ligand is labeled
with enzyme, using a molecular linker helps position
20   the receptor such that formation of the complex does
not denature the label, e.g., enzyme, for at least
several days.

5.1.1. RECEPTORS

25   One element of the receptor-ligand complex
is a receptor having one or more binding sites capable
of specifically binding to analyte, in which the
association constant of binding is high. The receptor
is also capable of binding to ligand with an
30   association constant of binding relatively low
compared to that of receptor binding to analyte.
Suitable receptors for use in release assays of the
invention include nucleic acid molecules (RNA or DNA),
antibodies (or a fragment of the antibody that
35   contains the analyte binding site for analyte and

ligand), cell surfac rec ptors (or a fragment of a
cell surface receptor that contains the binding site
for analyte and ligand), enzymes (or the substrate
binding site of an enzyme), lectins or any other
5 molecule or macromolecule capable of specifically
binding to and forming a stable low affinity complex
with a ligand and a stable high affinity complex with
an analyte. Antibodies and cell surface receptors are
preferred, with antibodies more preferred. In a
10 preferred embodiment, receptor is generated or
selected to be specific for the most unique epitope on
the analyte.

Various procedures known in the art may be
used for the production of antibodies to analytes of
15 interest. Such antibodies include but are not limited
to polyclonal, monoclonal, chimeric, single chain, Fab
fragments and an Fab expression library. For the
production of antibodies, various host animals may be
immunized by injection with a particular analyte or
20 analyte conjugated to an immunogenic carrier,
including but not limited to rabbits, mice, rats, etc.
Various adjuvants may be used to increase the
immunological response, depending on the host species,
including but not limited to Freund's (complete and
25 incomplete), mineral gels such as aluminum hydroxide,
surface active substances such as lysolecithin,
pluronic polyols, polyanions, peptides, oil emulsions,
keyhole limpet hemocyanin, dinitrophenol, and
potentially useful human adjuvants such as BCG
30 (bacilli Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies to analytes may be
prepared by using any technique which provides for the
production of antibody molecules by continuous cell
lines in culture or *in vivo*. These include but are
35 not limited to the hybridoma technique originally

described by Kohler and Milstein, (*Nature*, 1975, 256:495-497), the more recent human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today*, 4:72) and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention monoclonal antibodies specific to analytes may be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote at al., 1983, *Proc. Natl. Acad. Sci.*, 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.*, 81:6851-6855; Neuberger et al., 1984, *Nature*, 312:604-608; Takeda et al., 1985, *Nature*, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce analyte-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, *Science*, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to analytes.

Antibody fragments which contain sites
specific for analytes may be generated by known
techniques. For example, such fragments include but
are not limited to: the F(ab')$_2$ fragments which can be
5   produced by pepsin digestion of the antibody molecule
and the Fab fragments which can be generated by
reducing the disulfide bridges of the F(ab')$_2$
fragments.
Alternatively, polyclonal or monoclonal
10 antibody specific for an analyte of interest may be
obtained from commercial sources.
Receptor for binding analyte may be
purified, e.g., by affinity chromatography or low
affinity chromatography. Monoclonal antibody may also
15 be purified by protein A or anti-Ig chromatography.
Techniques for purifying polyclonal and monoclonal
antibodies are well known in the art. A heterogeneous
receptor preparation, such as polyclonal antibody, may
also be absorbed with a low concentration (e.g., 1% of
20 the receptor concentration) of ligand to remove any
receptors capable of binding ligand with high
affinity.

5.1.2. LIGAND

25   As used herein, the term "ligand" includes
molecules with limited cross-reactivity with analyte
for receptor binding. The term "reland" is used
herein interchangably with ligand. Reland is a term
coined by the co-inventors hereof to refer to a
30 release ligand. The ligand or reland binds receptor
with a low association constant relative to analyte,
e.g., preferably less than about 10%, more preferably
less than about 1%, and does not affect the stability
of an analyte-receptor complex. Ligand comprises an
35 analog of the analyte, including an epitope of the

analyte, a derivative of the analyte, a modified
analyte, or an isomer of th analyte. Preferably the
ligand differs from analyte at or near the receptor
binding epitope. These differences may include
5 stearic, configurational, conformational or ionic
changes. In another embodiment in which the receptor
is a nucleic acid, ligand is a nucleic acid
complementary to the "receptor", provided that the
degree of complementarity is not as high as the
10 complementarity of the receptor and the nucleic acid
analyte.

Analogs of analyte include the analogous
molecule from a related species of animal, where such
exists, e.g., sheep luteinizing hormone is an analog
15 of human chorionic gonadotropin (see Section 16.,
infra). Molecules prepared to structurally mimic the
analyte are also analogs for use as ligands. Such
structural mimics may be, but need not be, of the same
chemical nature as the analyte so long as the epitope
20 is chemically similar. Thus, for example, a peptide
may be an analog of a protein. A suitable analog will
simply share a receptor binding epitope or part of an
epitope of the analyte.

Derivatives of analyte may be prepared by
25 adding or deleting functional groups to the molecule.
Derivatives may also be natural metabolic products of
the analyte. One of ordinary skill will readily know
how to prepare or identify derivatives of analytes for
use in the invention. Preferably changes in molecular
30 structure of the analyte will alter the receptor
binding epitope composition or conformation in order
to decrease the binding affinity of receptor for
ligand.

Modified analyte includes analyte conjugated
35 to a carrier protein. Addition of bulky groups or

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ionic groups, such as aliphatic, aromatic or cyclic
molecules, to the analyte or to the protein can result
in decreased binding affinity for receptor due to
stearic and/or charge interference. Alternatively,
5 conjugation with a bulky group may cause a
conformational change in the receptor binding site of
ligand that decreases binding affinity. Chemical
modifications of organic molecules are well known in
the art and may be used to modify ligand. In a
10 specific Example, _infra_, an analyte, norcotinine,
modified by the presence of N-isopropyl or N-propyl
groups is a preferred ligand in an assay for cotinine.
        Isomers of analytes are molecules with the
same composition but different configuration.
15 Typically, isomers will have a different configuration
at a particular carbon center, e.g., cis versus trans,
D versus L. Isomers include diastereomers, which have
the opposite configuration at one or more chiral
centers, and include enantiomers of analytes. Since
20 biological binding interactions depend on configura-
tion as well as conformation and composition, use of
an isomer as ligand can result in much lower binding
affinity for receptor.
        Where the analyte is a protein, the ligand
25 may be prepared by recombinant DNA methods using site
directed or other mutagenesis techniques to alter the
amino acid sequence of the protein. These techniques
are well known to those skilled in the art (e.g., see
Zoller and Smith, 1984, _DNA_ 3:479-488; Oliphant et
30 al., 1986, _Gene_ 44:177; Hutchinson et al., 1986, _Proc._
Nat'l. Acad. Sci. U.S.A. 83:710). Polymerase chain
reaction (PCR) techniques are preferred for site
directed mutagenesis (see Higuchi, 1989, "Using PCR to
Engineer DNA," in _PCR Technology: Principles and_

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Applications for DNA Amplification, H. Erlich, ed.
Stockton Press, Chapter 6, pp. 61-70).

The ligand may further comprise a carrier
protein. The carrier protein may be an essentially
5 inert protein such as albumin or keyhole limpet
hemocyanin, but a carrier protein should not be the
same carrier used, if any, for immunization to produce
antibody to the analyte. An enzyme label may function
as a carrier protein.

10 Ligand may also comprise a linking group. A
linking group may space ligand from an enzyme label,
thereby preventing or slowing deactivation of the
enzyme after a long incubation with receptor. An
example of a spacer linking group is ε-aminocaproic
15 acid. Also, bulky linking groups may be used to
sterically hinder binding of receptor to ligand. An
example of a bulky linking group is p-aminobenzoic
acid.

Ligands may be evaluated to show their
20 suitability for use in a release assay. Ligand
evaluation assays include competition assays, binding
enhancement assays, direct assays, low affinity
chromatography, and microtiter plate release assays,
as well as the contemplated release assay itself.

25 Competitive assays useful for evaluating
potential ligands can be run in an ELISA format, but
any competitive assay technique may be used. As shown
in Example 16., <u>infra</u>, a ligand is much less effective
than analyte for inhibiting binding of receptor to
30 solid phase analyte. A molecule that is a poor
competitive inhibitor relative to analyte can be a
good choice for use as a ligand.

A low concentration of a suitable release
ligand may actually appear to enhance binding of
35 receptor to analyte in a competition assay. Thus in a

competition type assay with polyclonal antibody,
increased absolute signal in the presenc of the
molecule being tested indicates that molecule may be a
suitable release ligand.

5   When a direct assay is used to evaluate a
potential ligand, binding of receptor and ligand is
compared to binding of receptor and analyte. An ELISA
format is well suited for this type of assay, but
other assay formats can also be used. A ligand will
10 demonstrate no more than about 10%, and preferably
less than about 1%, of the binding activity of
analyte. Typically, in an ELISA, binding activity is
represented by titer, i.e., dilution or concentration
of receptor with a particular binding activity.
15 Alternatively, specific activity at the same receptor
concentration can be compared.

        If an analyte-specific receptor elutes under
mild conditions from an affinity chromatography column
prepared with ligand, that ligand may be a useful for
20 a release assay.

        In the microtiter plate method, ligand or
receptor is coated on the plate. When the microtiter
plate is coated with ligand, the ligand will
preferably comprise a protein to enhance binding to
25 the plate. The complementary binding partner
(receptor or ligand, respectively) is added and a
complex allowed to form. If the receptor-ligand pair
is suitable, release of receptor or ligand can be
detected in a sample supernatant when sample
30 containing analyte is added. As demonstrated in
Example 11., _infra_, a ligand-enzyme conjugate designed
for use in a homogeneous assay can be tested in a
microtiter plate assay.

35

5.2. RELEASE ASSAY FORMAT

The release assay comprising receptor, ligand and a means for detection of released, i.e., dissociated, receptor-ligand complex, has particular utility for analyzing a sample for the presence of a small molecule such as a drug metabolite. Detecting the presence of drugs or drug metabolites in a sample of body fluid from a patient is important for many applications. The presence of drugs or drug metabolites is useful in determining appropriate medical treatment, especially under emergency conditions. Detection of drugs or drug metabolites in a person is important in law enforcement, employment, schools and athletics. Samples may be from any source, but preferably are from an animal, and more preferably from a human. Samples may include but are not limited to be body fluid such as blood, plasma, serum, urine, saliva, bodily exudate, etc.

Analytes may be any antigen, but small analytes (MW of 100 to 1000 Daltons) are of primary interest. Such analytes include therapeutic drugs and metabolites thereof, illicit drugs and metabolites thereof, steroids, and peptide hormones. Nevertheless, release assays for larger molecules such as protein hormones, e.g., insulin, viral antigens, bacterial antigens, serum proteins, antibodies or any antigen of interest where detection of presence (or absence) of the analyte in a rapid, specific, sensitive assay is desirable are also contemplated.

In specific Examples infra, release assays for cotinine (a nicotine metabolite associated with smoking), benzoylecgonine (a cocaine metabolite), tetrahydrocannabinol (the narcotic agent of marijuana), thiazides (a class of diuretics), and beta-blockers are shown. Moreover, it will be

apparent that release assays are suitable to detect
any analyte of interest.

     For example, a release assay may be prepared
for HIV antibodies. A format for a release HIV assay
5 follows:

     1.   Generate antibodies to an HIV peptide
modified in such a way as to make it a release ligand,
e.g., by substitution of amino acids. This antibody
is then analogous to a ligand for purposes of the
10 assay since it is altered relative to the analyte
(anti-HIV antibody) of interest.

     2.   Conjugate the native HIV peptide
sequence to a protein such as albumin, and coat the
conjugate on plastic beads or wells of microtiter
15 plates. The peptide-protein conjugate acts as the
receptor for analyte for purposes of this assay.

     3.   Mix the receptor (conjugate) and the
ligand (antibody) so that complex is formed. The
ligand should be labeled with a marker e.g., a
20 fluorescent tag. Wash the plate after complex forms.

     4.   Add sample containing analyte (anti-HIV
antibodies) to the solid phase. The anti-HIV
antibodies displace the labeled antibody. Measure the
amount of released label.

25   Preferably the ligand antibody for an HIV
assay is a monoclonal antibody.

     The present invention provides for
positively detecting dissociation of a receptor-ligand
complex resulting in release of the labeled component.
30 Positive detection means that signal positively
correlates with the amount of analyte in a sample. A
positive correlation is advantageous because it is
psychologically satisfying that presence of signal or
increase in signal intensity indicates presence of
35 analyte in a sample. Results obtained by assay

methods in which detection of signal decreases when
analyte is present are susceptible to
misinterpretation. It is desirable to employ an
assay, such as that of the present invention, in which
5 only a positive result generates signal. Assays of
the invention are equally suitable for use both in a
laboratory by technical personnel as well as outside a
laboratory by both technical and non-technical
personnel.

10   Accordingly, the invention provides for
detecting release of a component of a receptor-ligand
complex. In a heterogeneous release assay (Section
5.2.2, infra), either receptor or ligand may be
labeled so that upon release in the presence of
15 analyte, the label signal is separated from reaction
products and is detected. In a homogeneous release
assay (Section 5.2.1, infra), it is preferable to
label ligand, but under certain circumstances, when
using a nonenzymatic label, receptor can be labeled.
20 For example, when a fluorescent label is used, ligand
may contain a fluorescence quencher. In the receptor-
ligand complex, the fluorescent signal is quenched,
and there is no detection of fluorescence until
dissociation occurs in the presence of analyte.
25 Suitable labels include enzymes, fluorophores,
chromophores, latex particles, colloidal gold, dyes
and chemiluminescent agents.

          The means for detecting dissociation of a
receptor-ligand complex depends in part on whether the
30 assay is homogeneous or heterogeneous, as described
below in sections 5.2.1. and 5.2.2.

          Once suitable binding receptor, release
ligand, and means for detecting a particular analyte
are chosen, the assay system must be optimized for use
35 with a particular sample matrix. A urine sample will

have different intrinsic characteristics than a sample
in aqueous buffer. The same is true for sample from
saliva, blood, plasma or serum, or any body fluid.
The assay may be optimized by varying reagent

5 concentration, buffer composition, release time,
detection time, baseline controls, and other
variables. These variables are well known in the art,
and it will be readily understood how to adjust them
for optimum assay specificity and sensitivity with a

10 particular assay matrix.

5.2.1. HOMOGENEOUS RELEASE ASSAYS

The release assay may be performed in
homogeneous liquid phase. Such an assay is preferred

15 because it can be performed in a single reaction
vessel, and thus is well suited for use in automated
analyzers.

In one embodiment, the ligand may be
conjugated to an enzyme label that retains a

20 detectable level of enzyme activity. A ligand-enzyme
conjugate is selected such that upon binding of
receptor to ligand, enzyme activity decreases. To
increase sensitivity, preferably one receptor binding
site is present on the enzyme, but more than one is

25 also acceptable.

When analyte is added to the receptor-ligand
complex, the complex dissociates into its receptor and
ligand components and released receptor binds analyte.
Upon release of the ligand-enzyme conjugate, enzyme

30 catalytic activity increases. This increase is
detected by measuring the rate of product formation.
Any enzyme-substrate system can be used, with the
proviso that no endogenous enzyme present in sample
will artificially increase the rate of product

35 formation. Preferably the enzyme is glucose-6-

phosphate dehydrogenase, and the reaction product is reduced nicotine-adenine dinucleotide (NADH), which can be detected by absorbance at 340 nm.

Alternatively, a receptor-ligand complex in
5 a homogeneous release assay may comprise a fluorescent label or chemiluminescent label attached to ligand and a fluorescence quencher attached to the receptor. The receptor may itself quench fluorescence. In the complex, the fluorescence or luminescence will be
10 quenched and no signal will be observed. However, upon dissociation of the receptor-ligand complex and release of receptor and ligand in the presence of analyte, quenching will diminish, and signal will be observed. Other proximity dependent signal
15 attenuators, such as fluorescence polarization, are known in the art, and can be adapted for use in a release assay. It will further be appreciated that the label may be on receptor and the quencher on ligand.

20

5.2.2. HETEROGENEOUS RELEASE ASSAYS

In another embodiment, a heterogeneous solid-phase/liquid-phase release assay is provided. In such an assay, either receptor or ligand is
25 irreversibly absorbed to a solid phase support. As used herein, the term "irreversibly absorbed" includes covalent, non-covalent and ionic association. Solid phase supports include plastic, polymer beads, glass beads, glass, silica gel, and membranes. In Examples
30 infra, solid phase supports are plastic microtiter plate wells and nitrocellulose membranes. However, the release assay is not limited to a particular choice of solid phase support and any solid phase support known in the art may be used.

35

The binding partner of the receptor or
ligand absorbed to the solid phase support, i.e., the
ligand or receptor respectively, is labeled, and a
stable complex comprising the labeled element and the
5   solid phase element is formed. Once a stable
receptor-ligand complex is formed, it can be exposed
to sample. If the analyte of interest is present in
the sample, the release reaction occurs and signal
from the label is detected in the liquid phase. The
10   extent of release, and thus the signal intensity in
the liquid phase, positively correlates with the
amount of analyte in the sample. The signal intensity
in the solid phase decreases inversely with the amount
of analyte in the sample (see Table 6).
15   Many labels can be used in the heterogeneous
release assay. Enzyme labels are practical, even for
a single vessel assay, because enzymes bound to a
solid phase can have 10-to 20-fold less catalytic
activity than the same enzyme in solution. Moreover,
20   as with homogeneous release, binding of receptor to
ligand-enzyme conjugate may reduce enzyme activity.
Other labels such as chromophores, fluorophores,
chemiluminescent agents, radioisotopes, chelating
complexes, dyes, colloidal gold and latex particles
25   can be detected in the liquid phase after release
reaction as increased optical density, fluorescence,
luminescence, radioactivity, color (for dyes), and
turbidity (for colloidal gold and latex particles),
respectively. Where the signal from label that
30   remains bound in the receptor-ligand complex cannot be
detected, the assay may be performed in a single
vessel.
             In a particular embodiment preferred for
non-laboratory settings, the presence of an analyte is
35   indicated by the appearance of a shape, i.e., a

letter, in a reaction field on a solid phase support. Accordingly, a reaction field comprising an indicator zone and a control zone is prepared on a solid phase support. The indicator zone comprises either

5 immobilized receptor or ligand, as provided by the heterogeneous assay format. A receptor-ligand complex in the indicator zone is sensitive to the release reaction. The control zone comprises a different receptor-ligand complex. The receptor-ligand complex

10 in the control zone is not susceptible to the specific release reaction, but will indicate non-specific release if conditions are such as to cause non-specific release.

In practice, contacting sample containing

15 the analyte of interest to the reaction field will result in a detectable release reaction in the indicator zone, and no reaction in the control zone. The release reaction is detected as formation of a contrasting zone corresponding to the indicator zone.

20 To accomplish this, label for both the release complex and the control complex is chosen to contrast with the solid support.

If there is no development of a contrast zone, the sample is negative. "Fade" of both the

25 indicator zone and control zone, i.e., release of label from both complexes, indicates a false positive reaction, inappropriate reaction conditions, and possible adulteration of the sample. In this way, the control zone provides a control for accurate assay

30 results.

Preferably different letters or symbols are used as the indicator depending on the analyte of interest. For example, indicator zone specific for cocaine use may be shaped like the letter "C"; an

35 indicator zone for marijuana use shaped like the

letter "M" (or "T" for tetrahydrocannabinol), and a
zone to indicate nicotine use shaped like the letter
"N".

5   The control zone comprising a complex
immobilized control receptor or ligand and labeled
ligand or receptor, respectively, is analogous to the
indicator zone, with the proviso that the control
complex is insensitive to the presence of analyte,
while the complex in the indicator zone is released in
10   the presence of analyte. For example, if the analyte
of interest is cotinine, the immobilized ligand is
cotinine coupled to bovine gamma globulin via an
aminocaproic acid linker, and the receptor is anti-
carboxy-cotinine labeled with blue latex, a suitable
15   control ligand is immobilized trinitrophenol (TNP)
conjugated to bovine serum albumin, and a suitable
control receptor is anti-TNP antibody, also labeled
with blue latex. In the presence of cotinine, labeled
anti-carboxycotinine would be released and a
20   contrasting zone develop. The TNP-anti-TNP complex is
unaffected by cotinine.

        It is clear that other receptor ligand
combinations will work equally well as control
complexes. For example, in an assay to detect
25   cotinine, a control complex could be immobilized
ecgonine-anti-benzoylecgonine. It is further
envisioned that a single solid phase support can
contain more than one detection field, since each
detection field is specific for a particular analyte
30   and insensitive for any other analyte. Thus, the
invention provides an assay for multiple analytes,
e.g., tetrahydrocannabinol, benzoylecgonine, and
cotinine, in a single format.

        Suitable labels for use in this assay
35   include but are not limited to colored latex

particles, dyes, colloidal gold, and enzymes that
catalyze production of an ins luble colored reaction
product when a white or light colored solid phase
support is used, and white latex when a dark solid
5   phase support is used. Preferably, colored latex
particles are used as labels. Also, any solid phase
support can be used in this embodiment, but plastic
and membranes are preferred.

In a preferred embodiment, such as described
10   in Section 15., *infra*, the solid phase support
comprising receptor-ligand complex and control complex
is provided dry.

The invention will be further clarified by
15   the following Examples, which are intended to be
purely exemplary of the invention and not as
limitations of the invention. Indeed, various
modifications of the invention in addition to those
shown and described herein will become apparent to
20   those skilled in the art from the description and
accompanying drawings. Such modifications are
intended to fall within the scope of the appended
claims.

25   6.   <u>EXAMPLE: SOLID PHASE RELEASE ASSAY FOR COTININE</u>

Cotinine and trans-3'-hydroxycotinine are
the major metabolites of nicotine (Langone et al.,
1973, *Biochem.* 12:5025-30; Jacob et al., 1991, *J.
Chromatography* 222:61-70; Neurath et al., 1987, *Int.
30   Arch. Occup. Environ. Health* 59:199-201). They appear
in urine in a 1:3 ratio (Newrath et al., *supra*). The
detection of cotinine in urine, serum or saliva is the
most commonly used biochemical method to determine
levels of exposure to nicotine (Fitzpatrick, 1991,
35   *C.N.N.* 11). Unlike other drugs of abuse, cotinine is

found in bodily fluids of non users due to passive
sm king. The rang of int rest for a cotinine assay
is from 0.010 μg/ml necessary for saliva and blood
testing, to <10μg/ml for urine of tobacco users

5   (Greenberg et al., 1984, *N. Engl. J. Med.* **310**:1075-78;
Matsukura et al., 1984, *N. Engl. J.Med.* **311**:828-31;
Sepkovicet al., 1985, *Am. J. Public Health* **75**:663-6;
Sepkovic et al., 1986, *J.A.M.A.* **256**:863; Jarvis et
al., 1987, *Am. J. Public Health* **77**:1435-8; Schepers

10 and Walk, 1988, *Arch. Toxicol.* **62**:395-7; Langone et
al., 1988, *J.I.M.* **114**:73-8).

        This example reports the use of three
different cotinine ligands in a heterogeneous solid
phase release assay. One ligand comprises a cotinine

15 metabolite directly bound to an inert carrier protein
(hydroxy-cotinine-BGG), another ligand comprises the
cotinine metabolite conjugated to the protein via a
bulky linker (hydroxycotinine-aminobenzyl-BGG) and a
third comprises the cotinine metabolite conjugated to

20 the protein via a spacer linker (hydroxycotinine-
aminocaproyl-BGG).

                6.1.   <u>MATERIALS AND METHODS</u>
        Trans-3'-hydroxycotinine was obtained

25 according to a published method (Jacob et al., 1990,
J. Med. Chem. **33**:1888). Succinic anhydride and
dimethyl-formamide (DMF) were obtained from Aldrich
Chemical Co. Pyridine, N-hydroxysuccinimide, 1-ethyl-
3-(3-dimethylaminopropyl) carbodiiumide (EDC), p-

30 aminobenzoic acid (PABA), ε-aminocaproic acid (ACA)
and bovine gamma globulin (BGG) were obtained from
Sigma.

35

6.1.1. PREPARATION OF HYDROXYCOTININE-BGG LIGAND

Step A. Hydroxycotinine hemisuccinate was prepared as follows. In a glass tube, 19 mg of trans-3'-hydroxycotinine were dissolved in 1 ml of DMF. In this solution 21 mg of succinic anhydride were dissolved. After adding 20 µl of pyridine, the tube was covered (PARAFILM®) and incubated overnight at 37°C.

Step B. After equilibration at room temperature (r.t.), 14 mg of N-hydroxysuccinimide and 23 mg of EDC were dissolved in the cotinine solution. The solution was covered (parafilm) and incubated 3 hours at room temperature to activate the cotinine hemisuccinate.

Step C. Activated cotinine hemisuccinate (400 µl of the solution from step B.) was added to the solution of 6 mg BGG dissolved in 1 ml of PBS. The mixture was gently mixed and incubated 1 hour at room temperature, then overnight at 4°C. The resulting conjugate was dialyzed against 6 changes of PBS for 48 hours.

6.1.2. PREPARATION OF HYDROXYCOTININE-AMINOBENZOYL-BGG LIGAND

Step D. In 0.6 ml of PBS were dissolved 6 mg of PABA. To this solution were added 400 µl of activated cotinine hemisuccinate prepared according to step B., supra. The resulting solution was mixed well and incubated for 1 hour at room temperature and overnight at 4°C.

Step E. The hydroxycotinine-hemisuccinate-PABA was brought to room temperature and activated by addition of 0.5 ml of 14 mg/ml N-hydroxysuccinimide in DMF and 0.5 ml of 24 mg/ml EDC in DMF. The mixture was incubated 3 hours at room temperature. To 6 mg of

BGG in 2 ml of PBS wer added 400 µl of activated
cotinine-PABA. The resulting solution was mixed
gently and incubated 1 hour at room temperature and
overnight at 4°C. The resulting conjugate was
5   dialyzed against 6 changes of PBS over 48 hours.

6.1.3. PREPARATION OF HYDROXYCOTININE-AMINOCAPROYL-BGG   LIGAND

Step F.   To a solution of 4 mg ACA in 0.6
10 ml of PBS were added 400 µl of the activated cotinine
succinate solution prepared according to step B.,
supra. The resulting solution was mixed very well and
incubated 1 hour at room temperature and overnight at
4°C.

15 Step G.   The hydroxycotininehemisuccinate-
ACA solution was brought to room temperature and
activated by addition of 0.5 ml of 14 mg/ml N-
hydroxysuccinimide in DMF and 0.5 ml of 24 mg/ml EDC
in DMF. The resulting mixture was incubated for 3
hours at room temperature. To 6 mg of BGG in 2 ml of
20 PBS were added 400 µl of activated cotinine-ACA. The
resulting solution was mixed gently and incubated at
room temperature for 1 hour, then overnight at 4°C.
The resulting conjugate was dialyzed against six
changes of PBS over 48 hours.
25

6.1.4. ANTISERUM BINDING REAGENT

Antiserum to carboxycotinine was obtained as
follows: 320 mg of keyhole limpet hemocyanin (KLH)
protein (Sigma) were dissolved in 40 ml of deionized
30 water. To this were added 300 mg of
trans-4'-carboxycotinine (Aldrich) with mixing until
it was dissolved. Then 300 mg of EDC were added to
the reaction mixture with stirring, which was
continued overnight at room temperature. The KLH-
35 carboxycotinine conjugate was dialyzed for 8 hours at

2-8° against phosphate buffered saline. The dialysis fluid was changed once after 4 h urs.

Rabbits were immunized with the immunogen in Freund's adjuvant, with multiple injections over several months according to standard protocols. Test bleedings were made at defined intervals, and increases in antibody titer measured using an enzyme immunoassay for cotinine. Measurements of antibody affinity and cross-reactivity were also performed. When these assays indicated satisfactory antibody performance, rabbits were bled and sera were isolated and pooled. Antiserum was stored at -40°C.

The IgG fraction was separated from serum by ammonium sulfate precipitation. An immunoaffinity chromatography column was prepared by coupling succinylated hydroxycotinine through its carboxyl group to amino-sepharose 4B (see section 9., *infra*). The affinity purified antibody was labeled with horseradish peroxidase using the sodium-m-periodate method.

Antisera prepared against 4'-carboxycotinine, which is conjugated to carrier protein at the 4' position, were expected to bind cotinine conjugated to protein at the 3' position with lower affinity.

6.1.5. ASSAY

Microtiter plates were coated overnight with 100 μl of each ligand at 1 μg protein per ml of PBS. Dried plates were incubated for 1 hr with 100 μl of enzyme-labeled antibody. Excess antibody was washed out and 90 μl of distilled water and 10 μl of urine sample or standard were added to each well. After 2 minutes of shaking, the supernatant was transferred to uncoated wells. Released label in the supernatant

was quantitated after reaction with TMB substrate for
15 minutes by measurement of O.D. at 450 nm.

6.2. RESULTS

5   Release of labeled antibody complexed to
solid phase ligand was detected in supernatant when
free cotinine was present in sample. A comparison of
release from solid phase hydroxycotinine-PABA-BGG and
solid phase hydroxycotinine-ACA-BGG indicates that
10 after 2 minutes of exposure free cotinine induces the
release of anti-cotinine antibody bound to both
antigens in a concentration dependent manner (Figure
1). The results indicate that release from
hydroxycotinine-ACA-BGG is slightly more efficient.
15   Enhanced release reactions were observed for
release when the release portion of the assay is
extended to 10 minutes (Figure 2). Increases in A_{450}
could be observed at as low as 0.5 μg/ml cotinine.
Again, release from hydroxycotinine-ACA-BGG was more
20 efficient. A comparison of release after 2 minutes of
incubation with sample in urine and synthetic matrices
(Figure 3) indicates that (i) assay sensitivity is
greater in synthetic matrix than in urine matrix; and
(ii) that hydroxycotinine-ACA-BGG acts more
25 efficiently for release of anti-cotinine antibody.

6.3. DISCUSSION

These results show that a release assay
indicates the presence of free cotinine in a sample of
30 urine or an artificial urine matrix. Assay plates
prepared by coating the microwells with cotinine
ligand conjugated at the 3' position to a protein and
forming a complex with antibody specific for cotinine
conjugated to the immunogenic carrier protein at the
35 4' position, the detection of free cotinine in sample

can be completed using this assay in under 30 minutes.
Comparison of Figures 2 and 3 shows that rel ase can
be observed as soon as 2 minutes after sample is
added.

5   Subtle differences in binding affinity
affect the sensitivity of a release assay. Release
from the hydroxycotinine-aminocaproic acid-bovine
gamma globulin ligand gave slightly more sensitive
readings than release from hydroxycotinine-

10 aminobenzoyl-BGG. These results demonstrate how
exploitation of differences in binding affinity allows
development of sensitive immunoassays.

         7. EXAMPLE: MICROTITER PLATE METHOD
             OF ASSAYING LIGAND-PROTEIN CONJUGATES

15
         A solid phase assay provides a method to
compare ligand-protein conjugate preparations for use
in release assays, and to determine whether a
particular ligand binds analyte-specific antibody with

20 sufficiently low affinity. The present example
reports results with a cotinine ligand.

         7.1. MATERIALS AND METHODS
         Reagents, hydroxycotinine-p-amino-benzoic

25 acid-bovine gamma globulin (BGG) and hydroxycotinine-
ε-aminoncaproic acid-BGG were prepared as described in
section 6.1., supra.

         7.1.1. ASSAY PROTOCOL

30   Microtiter plates were coated with cotinine-
ligand as described in Section 6.1.5., supra. The
wells were washed twice with wash buffer and 100 μl of
anti-cotinine-peroxidase conjugate (Section 6.1.4.,
supra) were added to each well. The plate was

35 incubated 60 min at r.t. and washed twice with wash

buffer. To each well were added 100 µl of wash buffer
and 10 µl of sampl . Samples consisted of 0, 0.5, 2.0
and 10 µg/ml cotinine in urine, and two negative and
two positive urine samples. The plate was incubated
5   10 minutes at r.t. with shaking, and 100 µl of
supernatant were removed and transferred to replicate
wells of on uncoated microtiter plate. To the wells
containing supernatant were added 100 µl of 2x TMB
substrate solution. The plate was incubated 3 min at
10   r.t., and then the reaction stopped by addition of 50
µl 2N sulfuric acid to each well. Absorbance at 450
nm was measured.

7.2. RESULTS

15   Presence of cotinine in sample caused
release of labeled anti-cotinine antibody from the
cotinine-ligand solid phase. The A_{450} value of
supernatant correlated positively with the amount of
free cotinine present in cotinine-spiked urine
20   samples. Furthermore, the results with negative and
positive urine samples demonstrate the usefulness of
the assay to detect cotinine in urine. The results
are summarized in Table 1.

25

30

35

TABLE 1.   COTININE-INDUCED RELEASE FROM
           COTININE-BGG-CONJUGATES.

COTININE CONC (μg/ml)	A_{450}	
	COTININE-PABA-BGG	COTININE-ACA-BGG
0	0.637	0.589
0.5	0.658	0.721
2.0	0.946	0.986
10.0	2.247	2.251
NEG. URINE 1	0.515	0.585
NEG. URINE 2	0.368	0.420
POS. URINE 1	1.161	1.269
POS. URINE 2	1.077	1.198

7.3 DISCUSSION

These results show that a microtiter plate assay provides a convenient support for demonstrating the usefulness of a particular ligand-protein conjugate for a release assay. In this assay use of a bulky or spacer linkers in the ligand made little difference in the results.

8. EXAMPLE: HOMOGENEOUS RELEASE ASSAY FOR COTININE

The release system provides improved sensitivity and specificity over previous assays by taking advantage of the specificity of dissociation reactions. Dissociation reactions offer the advantage that they are less subject to interfering substances.

The present example demonstrates

(1) preparation of cotinine ligands labeled with the enzyme glucose-6-phosphate dehydrogenase;

(2) inhibition of enzymatic-activity of the enzyme-cotinine conjugate in the presence of anti-cotinine;

(3) displacement of anti-cotinine from the enzyme-
cotinine conjugate in the pres nce of cotinine; (4) an
automated assay for release of antibody from ligand,
particularly after to 22 hrs of complex incubation;
5 and (5) stabilization of an antibody-ligand complex
for up to 6 days.

8.1.   MATERIALS AND METHODS
      The following reagents were used: trans-4'-
10 carboxycotinine, N-hydroxysuccinimide, 1,3-
dicyclocarbodiimide, and dimethylformamide (DMF), all
from Aldrich Chemical Co.; glucose-6-phosphate
dehydrogenase (Beckman, 367 IU/mg protein); glucose-6-
phosphate and beta-nicotinamide adenine dinucleotide
15 reduced form (NAD), both from Sigma Chemical Co.; and
carbinol [2-(2-ethoxyethoxy)ethanol], from Aldrich
Chemical Co.
      Preparation of cis-hydroxycotinine-glucose-
6-phosphate dehydrogenase is described in Section
20 10.1.3., _infra_.

8.1.1. ASSAY PROCEDURE
      Assay buffer was 0.05 M Tris-HCl, pH 7.8,
equilibrated at 37°C.   To 150 μl of assay buffer were
25 added 10 μl of cotinine-enzyme conjugate and 1 μl of
anti-cotinine antibody (Section 6.1.4., _supra_), and
the solution incubated 10 minutes at 37°C.   To this
solution were added 10 μl of sample containing
cotinine.   The reaction was started by addition of
30 20 μl of glucose-6-phosphate/NAD, and absorbance at
340 nm read every 1 minute.
      A control reaction in the absence of
antibody was prepared by adding 10 μl of enzyme-
cotinine conjugate to 160 μl of assay buffer, and
35 equilibrating at 37°C for 10 min.   To start the

reaction, 20 μl of glucose-6-phosphate/NAD were added, and A_{340} was measured at intervals of 1 minute.

Cotinine-enzyme conjugate was prepared at 1 mg/ml of protein and diluted 500-fold to a final

5   concentration of 2.0 μg/ml. The anti-cotinine antibody was present in stock solution at an approximate concentration of 15 mg/ml. The glucose-6-phosphate-NAD solution was prepared by mixing 3 volumes of 0.11 M glucose-6-phosphate with 2 volumes

10 of 0.1 M NAD.

8.1.2. AUTOMATED ASSAY PROCEDURE

Reagent A was prepared with an anti-cotinine anti-serum (1:150 dilution), cotinine-glucose-6-

15 phosphate dehydrogenase conjugate, and 0.0015% Kathon CG in 0.05 M Tris-HCl, 0.005 M $MgCl_2$, pH 7.8. Reagent B contained 6.2 mg/ml glucose-6-phosphate and 13.3 mg/ml NAD.

Reagent reservoirs on an EPOS automated

20 analyzer (Eppendorf) were filled with reagents A and B. Reagents A (0.2 ml) was incubated for 0, 18 or 22 hrs prior to addition of sample (0.01 ml). Samples, including standards, were pipetted into the mixture of reagents A. Reagent B (0.05 ml) was added, and

25 absorbance at 340_{nm} was measured at 0 and 5 min.

8.2. RESULTS
8.2.1. REDUCTION AND INHIBITION OF ENZYME ACTIVITY

30 Enzyme activity of cis-hydroxycotinine-enzyme conjugate was assayed in the presence and absence of anti-cotinine antibody. The results are summarized in Figures 5 and 6.

Conjugation of cotinine to enzyme results in

35 decreased enzyme activity. Addition of anti-cotinine antibody to the enzyme-cotinine conjugate further

reduced (inhibited) enzyme activity (Figur 4).
Inhibition of enzyme activity by antibody binding is
completely reversed by the addition of 10 µl sample of
4 µg/ml cotinine (Figure 5).

5

8.2.2. AUTOMATED ENZYME ASSAY

Samples spiked with known amounts of
cotinine, or which were known to be positive for
cotinine, were assayed 0, 18 and 22 hrs after mixing
10 the antibody/ligand-enzyme conjugate. The assay was
performed as described in Section 8.1.2. The results
indicate that although long incubation times result in
somewhat decreased absolute rate of change, presence
of cotinine in the sample could be detected at even
15 the lowest concentration. These results are shown in
Table 2.

TABLE 2. AUTOMATED COTININE RELEASE ASSAY.

COTININE CONC. µg/ml	OD Rate Change (mAU per min) TIME OF PREINCUBATION OF REAGENT A PRIOR TO ADDITION OF STANDARDS OR SAMPLES		
	0 HOURS	18 HOURS	22 HOURS
0	102.1	101.1	102.4
0.5	116.3	108.5	109.1
1.0	123.0	111.0	111.3
2.0	127.3	114.5	114.8
4.0	132.5	118.2	118.2
8.0	138.0	121.8	121.7
POSITIVE #1	131.8	118.5	117.7
POSITIVE #2	130.0	117.7	117.9
POSITIVE #3	110.6	105.7	104.6
NEGATIVE #1 (T.D.)	103.0	104.7	101.2

These data indicate that the mixed reagent A
35 remains stable for as long as 22 hours. A statistical

analysis of the estimated amount of cotinine in the positive and negative urine samples bears out this conclusion (Table 3).

5   TABLE 3.   STATISTICAL ANALYSIS OF DATA
             FROM THEAUTOMATED ASSAY.

REAGENTS A PRE-INCUBATION TIME	STANDARD CURVES		ESTIMATED CONCENTRATION OF COTININE IN SAMPLES (μg/ml)			
	SLOPE	INTERCEPT	#1	#2	#3	T.D.*
ZERO TIME	0.9939	0.00838	3.55	2.80	0.221	0.08
18 HOURS	1.0583	-0.127	4.27	3.63	0.309	0.25
22 HOURS	1.0649	-0.142	3.57	3.72	0.211	0.101

* Negative sample.

15
        Table 3 shows that after 0, 18, or 22 hours of preincubation, agreement as to the amount of cotinine present in a sample is reasonably good. For example, the estimated concentration of cotinine in positive sample #1 ranges between 3.6 and 4.3 μg/ml.
20   Similar data are obtained for all four samples. Thus although the absolute absorbance change is greater after <1 hours of incubation time, all three curves provide good data for estimating concentration by extrapolation from the standard curve (Figure 6).
25

        8.2.3.   STABILIZATION OF A RECEPTOR-LIGAND
                 COMPLEX IN HIGH SALT SOLUTION

        Antibody-ligand complex was formed in 0.5 M
30   Tris-HCl, pH 7.9, containing 3 mM magnesium chloride and 0 or 5% sodium chloride. The assay was run using the format described in Section 8.1.1. The homogeneous release assay for cotinine was tested for receptor-ligand complex stability for up to six days.
35   Results are shown in Table 4.

TABLE 4.   RELEASE ASSAY AFTER LONG TERM INCUBATION
           OF AN ANTIBODY-LIGAND COMPLEX.

Cotinine conc. (µg/ml)		Rate of OD Change (mAU/min)	
		0% NACL	5% NACL
0	Day 0	12.09	16.6
0.5		13.15	17.63
1		13.43	18.96
2		13.85	18.3
Delta*		1.76	1.7
0	Day 1	7.02	9.67
0.5		7.5	10
1		7.7	11
2		7.8	11
Delta*		0.78	1.33
0	Day 6	7.3	9.1
0.5		7.8	9.3
1		7.6	9.6
2		7.9	10.7
Delta*		0.6	1.6

*   Delta is the enzyme activity in the presence of
     2 µg/ml cotinine with the baseline (activity in
     the absence of cotinine) subtracted.

        The data show that 5% sodium chloride
stabilized both enzyme activity and the antibody-
ligand complex in releasable form. Although the
magnitude of the maximum enzyme activity decreased
with time even in the presence of NaCl from 18.3
(day 0), to 11 (day 1), to 10.7 (day 6), the enzyme
activity with baseline subtracted remains fairly
constant at 1.7, 1.33 and 1.6 for days 0, 1 and 6,
respectively. The samples incubated in the absence of
NaCl did not remain stable.

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9. **EXAMPLE: PREPARATION OF LOW AFFINITY ANTIBODIES FOR USE IN RELEASE ASSAYS**

Low affinity chromatography provides a useful method to purify antibodies or other receptors, and may also indicate suitable ligands. In particular, receptor elution under mild conditions results in much higher yields than conventional affinity chromatography, e.g., close to 100%. The mild conditions preserve antibody from irreversible denaturation and extend the column life. An affinity column prepared with a potential ligand indicates that the ligand can be used in a release assay with that receptor. This Example demonstrates purification of anti-cotinine antibodies by a ligand, trans-3'-hydroxycotinine, affinity column.

9.1. MATERIALS AND METHODS

9.1.1. PREPARATION OF IMMUNOABSORBENT

Cotinine hemisuccinate was prepared by dissolving 19 mg of trans-3'-hydroxycotinine in 1 ml of dimethylformamide (Aldrich). 21 mg of succinic anhydride (Aldrich) were added, followed by 15 μl of pyridine (Aldrich). The mixture was incubated overnight at 45°C.

Iminodipropylamine-Sepharose was prepared as followed. Cyanogen bromide (CNBr) activated Sepharose 4B (Sigma), 1 gm, was hydrated and washed several times with 0.1 M carbonate buffer, pH 9.5. Three ml of 0.1 M 3,3'-iminobispropylamine (Sigma) were added to 3 ml of CNBr-Sepharose gel and the mixture mixed overnight at room temperature. The amino Sepharose was washed with a buffer consisting of 0.1 M sodium carbonate, pH 8.5, 0.1 M sodium bicarbonate, pH 8.5, and 0.5 M sodium chloride until no reactivity with trinitrobenzene-sulfonic acid with free amino groups was detected.

Hydroxycotinine h misuccinate succinic ester
was prepared by adding to 1 ml of cotinine
hemisuccinate 14 mg of N-hydroxysuccinimide (Sigma)
and 23 mg of 1-ethyl-3-(dimethylaminopropyl)
5   carbodiimide (Sigma) and incubating at room
temperature for 3 hours.

Hydroxycotinine-Sepharose was prepared by
mixing 0.45 ml of hydroxycotinine-succinyl ester with
1 ml of iminodipropylaminosepharose and 0.6 ml of 0.2
10   M sodium bicarbonate. The mixture was gently shaken
for 3 hours at room temperature and kept at 4°C
overnight.

9.1.2. AFFINITY CHROMATOGRAPHY

15   A small column was packed with the
hydroxycotinine-Sepharose gel, and the column washed 3
times with 1 ml of 0.5 M sodium chloride, and then
with phosphate buffered saline (PBS).

A portion of anti-cotinine antiserum
20   produced by injecting rabbits with carboxycotinine
(section 6.1.4., supra) was diluted 1:1 with PBS, and
the whole mixture (2.1 ml) applied to the column. The
material was allowed to equilibrate for 10 minutes,
and then the column was eluted sequentially with PBS,
25   0.1 M acetate buffer, pH 4.0, and 0.1 M citrate
buffer, pH 2.3. The column was then washed with 1 M
sodium chloride followed by PBS. Protein
concentration was determined on fractions using Pierce
Coomassie blue reagent, and antibody activity by
30   determining reactivity on plates coated with cotinine
in an ELISA assay. Orthophenylene-diamine (OPD) and
peroxide were used as indicator and substrate
respectively for peroxidase.

35

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9.2. RESULTS

Although most protein eluted in early fractions (void volume) from the affinity column, antibody activity was found in later fractions.
5 Fraction 14 contains antibodies with high affinity for cotinine. The antibody yield was greater than 80%, which is very high. Furthermore, the specific activity of the low affinity purified antibody as demonstrated by the number of tests performed was
10 better than with whole sera or the IgG fraction thereof (data not shown).

9.3. DISCUSSION

Low affinity chromatography provides a
15 superior way to obtain low affinity antibodies for use in release assays with that ligand. Low affinity antibodies elute from an immuno-affinity chromatography column earlier, under gentler conditions, than high affinity antibodies. Less
20 stringent conditions are sufficient to elute low affinity antibodies from a solid phase chromatographic support only if the antibody-ligand interaction is itself of sufficiently low affinity.

Elution under neutral or mild conditions
25 provides the additional benefit of reducing or preventing antibody denaturation.

10. PREPARATION OF LOW AFFINITY ANALOGS OF COTININE AND THEIR EFFICACY IN A RELEASE ASSAY

30 Ligands can be prepared by using analogs or stereoisomers of analogs of the analyte, thus introducing a configurational or constitutional difference that can result in lower affinity binding to analyte-specific antibody.
35

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In the present example, anti-cotinine antibody prepared from rabbits immunized with carb xycotinine conjugated to KLH (see Section 6.1.4., _supra_) is shown to have useful release properties from

5 trans-3'-hydroxy-cotinine conjugates of horseradish peroxidase in the presence of free cotinine. The optical isomer of trans-3'-hydroxycotinine is cis-3'-hydroxycotinine, and this is also shown to be a superior ligand.

10

10.1. MATERIALS AND METHODS

10.1.1. EXTENSIVE MODIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE WITH TRANS-3'-HYDROXYCOTININE TO FORM A LIGAND

Eleven mg of trans-3'-hydroxycotinine were
15 dissolved in 0.29 ml of dimethylformamide; 12 mg of succinic anhydride were added to the mixture. When all solids had dissolved, 9 μl of pyridine were added, and the tube was capped tightly and incubated at 37°C overnight. To the hydroxycotinine succinate mixture
20 were added 7 mg of N-hydroxysuccinimide and 12 mg of N,N'-dicyclohexylcarbodiimide. This mixture was incubated for 2 hours at room temperature.

Three tubes were prepared, each containing 1 ml of 0.1 M carbonate buffer, pH 9.0 and 2.8 mg
25 glucose-6-phosphate dehydrogenase. To the first tube were added 35 μl of the activated trans-hydroxycotinine over a period of 1 hour, in aliquots of 5 μl at 10 minute intervals. This preparation was designated as RL-24. To the second tube were added 70
30 μl of activated trans-hydroxycotinine, in the same regimen as above. This preparation was designated as RL-25. To the third tube were added 105 μl of activated trans-hydroxycotinine, in the same regimen as above, in 15 μl aliquots. This preparation was
35 designated as RL-26.

Fifteen minutes after the last addition of activated trans-hydroxycotinine, samples were transferred to dialysis bags and dialyzed at 2-8°C against 0.05 M Tris buffer, pH 7.8. Dialysis was continued for approximately 44 hours with several changes of fluid.

10.1.2. LESS EXTENSIVE MODIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE WITH TRANS-HYDROXYCOTININE TO FORM A LIGAND

9.5 mg of trans-3'-hydroxycotinine were dissolved in 0.25 ml of dimethylformamide. Then 6 mg of succinic anhydride were added to the mixture and mixed until dissolved. 7.5 μl of pyridine were added, and the tube was capped tightly and incubated at 37°C overnight.

To this mixture were added 6 mg of N-hydroxysuccinimide and 10 mg of N,N'dicyclohexyl-carbodiimide. The mixture was incubated for 45 min at room temperature with stirring.

Two tubes were prepared, each containing 1 ml of 0.1 M carbonate buffer, pH 9.0, and 2.8 mg glucose-6-phosphate dehydrogenase. To the first tube were added 25 μl of the activated trans-hydroxycotinine over a period of 1 hour, in aliquots of 5 μl at 10 minute intervals. This preparation was designated as RL-29. To the second tube were added 70 μl of activated trans-hydroxycotinine in the same regimen as above, in 10 μl aliquots. This preparation was designated as RL-30.

The preparations were dialyzed overnight in the cold against 0.05 M Tris buffer, pH 7.8.

10.1.3. A CIS-3'-HYDROXYCOTININE LIGAND

47.5 mg of cis-3'-hydroxycotinine were dissolved in 0.4 ml of pyridine. To this solution

were added 10 mg of succinic anhydride and the mixture
stirred until dissolved. The mixture was incubated
overnight at 37°C and then held at room temperature.

                    Cis-hydroxycotinine hemisuccinate was

5   separated from the reaction mixture with preparative
thin layer chromatography on silica gel using a
solvent system composed of benzene:methanol:ammonium
hydroxide, 10:3:0.5. The cis-hydroxycotinine was
visualized with a UV lamp. The desired product was

10 isolated as follows: the area on the chromatogram
containing the cis-hydroxycotinine hemisuccinate was
scraped off and placed in a tube. One ml of methanol
was added and the tube shaken by hand while the
contents were mixed with a glass spatula. The tube

15 was centrifuged briefly at 1500 rpm. The supernatant
was collected, and the silica gel washed three times
with 0.5 ml of methanol. Following centrifugation,
the supernatants were collected and combined with the
original supernatant. The combined supernatants were

20 evaporated to dryness under a stream of nitrogen. The
cis-hydroxycotinine hemisuccinate was reconstituted in
0.6 ml of methanol. After spectrophotometry it was
determined that 5.28 mg of product had been obtained.
The material was then evaporated to dryness again.

25   The cis-hydroxycotinine hemisuccinate was
dissolved in 280 μl of dimethylformamide. Eight mg of
N-hydroxysuccinimide and 12 mg of dicyclohexylcarbo-
diimide were added, and the mixture stirred until all
solids had dissolved. The mixture was incubated for 1

30 hour at room temperature with stirring.

       A tube was prepared containing 1 ml of 0.1 M
carbonate buffer pH 9.0 and 2.8 mg glucose-6-phosphate
dehydrogenase. To this tube were added seven 10 μl
aliquots of the cis-hydroxycotinine hemisuccinate over

35 a period of 1 hour, and mixing continued for 15

minutes after the last addition. The mixture was
transferred to a dialysis bag and dialyzed overnight
in the cold against 0.05 M Tris-HCl pH 7.8. Dialysis
was continued for an additional 26 hours with several
5   changes of buffer. This conjugate was designated as
RL-34.

10.1.4. PREPARATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE-CARBOXYCOTININE LIGAND

10   Carboxycotinine was conjugated to glucose-6-
phosphate dehydrogenase according to the following
procedure:
         To 1 ml of 0.1 M sodium carbonate buffer, pH
9.0, were added 0.43 ml of glucose-6-phosphate
15   dehydrogenase (2.8 mg), 20 mg of NADH (disodium salt),
10 mg of glucose-6-phosphate, and 300 μl of carbinol.
The solution ("enzyme solution") was stored at 4°C to
chill.
         To an empty test tube were added 22 mg of
20   carboxycotinine, 11.5 mg of N-hydroxysuccinimide, 20.6
mg of dicyclohexylcarbodiimide, and 1.0 ml of
dimethylformamide. This mixture was left at room
temperature for 1 hour to allow the activated cotinine
ester to form. After 1 hour, 10 μl of the reaction
25   mixture were added to the cold enzyme solution at 15
minute intervals until a total of 70 μl were added (90
minutes total). Fifteen minutes after the final
addition of reaction mixture, the modified enzyme was
dialyzed against five changes of 1 liter each of
30   0.055 M Tris-HCl buffer, pH 7.9, for at least three
hours each.

35

10.1.5. ASSAY IN MICROTITER PLATES

Strip microtiter plates were coated by addition of 100 μl of each of the conjugates at a protein concentration of 1 μg/ml to each well. The strips were covered with parafilm and incubated overnight at room temperature. The well contents were discarded, and wells washed twice with wash buffer. The wells were then incubated with 100 μl of antibody-peroxidase conjugate for 1 hour at r.t. Unbound conjugate was discarded and the wells washed twice with wash buffer. To each well were added 100 μl of wash buffer and 10 μl of cotinine standards, and the plates were mixed for 2 minutes. After mixing, 100 μl of liquid from each well was transferred to wells in uncoated plates, and 100 μl of tetramethylbenzidine (TMB) reagent were added. The plates were incubated for 7 minutes at room temperature, after which the reaction was stopped by addition of 50 μl of 2N sulfuric acid to each well. Absorbance was measured at 450 nm.

Residual peroxidase-antibody conjugate remaining bound to wells after release was measured by adding 200 μl of TMB to each well. After a 7 minute incubation, the reaction was stopped by addition of 50 μl of 2N sulfuric acid, and absorbance at 450 nm was measured.

A previously made conjugate of carboxycotinine-glucose-6-phosphate dehydrogenase was also assayed by this method.

10.2. RESULTS

The results of assays with various hydroxycotinine-glucose-6-phosphate dehydrogenase conjugates are shown in Tables 5, 6 and 7.

TABLE 5. RELEASED ANTIBODY-PEROXIDASE CONJUGATE (A_{450}).

CONJUGATE # COTININE STANDARD (μg/ml)	24	25	26	29	30	CARBOXY COTININE CONJU-GATE
0	0.971	1.049	1.030	0.442	0.662	0.498
0.5	1.911	2.071	1.990	0.753	1.051	0.505
2.0	2.529	2.739	2.635	0.948	1.436	0.526
10	2.804	>3	>3	1.355	1.974	0.728

TABLE 6. ANTIBODY-PEROXIDASE CONJUGATE (A_{450}) REMAINING ON WELLS.

CONJUGATE # COTININE STANDARD (μg/ml)	24	25	26	29	30	CARBOXY COTININE CONJUGATE
0	2.474	2.514	2.513	1.893	2.097	2.707
0.5	2.403	2.368	2.440	1.776	1.937	2.746
2.0	2.213	2.347	2.360	1.739	1.879	2.730
10	2.051	2.253	2.298	1.695	1.790	2.786

TABLE 7. RELEASE ASSAY WITH CIS-HYDROXYCOTININE.

COTININE CONC. (μg/ml)	Absorbance at 450 nm	
	RELEASED CONJUGATE	REMAINING CONJUGATE
0	0.475	2.055
0.5	1.591	1.921
2.0	2.268	1.730
8.0	2.683	1.210

Release of enzyme-antibody conjugate activity is also shown graphically in Figure 7. These results clearly indicate that use of modified analog as ligand provides the necessary difference in affinity for a release-type assay.

The cis-hydroxycotinine-gluc se-6-phosphate
dehydrogenase ligand was also tested in a homogeneous
release assay such as described in Section 8.1.2.,
supra. After a 10 minute incubation of 10 μl of
5   antibody binding reagent with a 10 μl of ligand in 140
μl of Tris buffer, 10 μl of sample were added. NAD
and glucose-6-phosphate substrate (20 μl) were added
after an additional 5 minute period, and A_{340} read at 0,
2, 4, 8, 12, 16 and 20 minutes. Samples containing
10 cotinine showed a more rapid increase in absorbance
than sample with no cotinine. The results are shown
in Table 8.

TABLE 8. HOMOGENEOUS RELEASE WITH CIS-HYDROXYCOTININE.
15

Cotinine conc. (μg/ml)	OD Rate Change (mA/min)
0	8.8
0.5	17.5
4.0	20.0

20

These results indicate that a microtiter
plate format is ideal for screening for suitable low
25 affinity ligands and ligand-enzyme conjugates. A
ligand found to be suitable in a microtiter plate
release assay can be used in a homogeneous release
assay as well.

30   11. EXAMPLE: RELEASE IMMUNOASSAY FOR COTININE
In this Example 11, cotinine release assays
using N-propylcarboxynorcotinine and N-
isopropylcarboxynorcotinine as very low affinity
relands is described. Cis 3'-hydroxycotinine was also
35 used as a reland. The release assays were performed

in both homogeneous (which can be compared to, but
which is demonstrated to be superior than, the EMIT™
System (Rubenstein et al., 1972, Biochem. Biopys. Res.
Comm. 47: 846)) and heterogeneous (microtiter plate,
5   ELISA format) formats and compared to a conventional
associative assay for cotinine. The results
demonstrated that the release assays of the invention,
based on dissociation, are more precise and exhibit
less interference from cross-reactivity than known
10   assays based on association. Moreover, the release
assay of the invention has a standard curve that is
linear, r 0.999, over a >10,000 fold range.
         The following materials and methods sections
set forth general descriptions of the reagents
15   prepared and used in the assays, as well as the
methods employed. The specific ligands used in each
assay are identified in the results sections, and
unless otherwise specified is N-
isopropylcarboxynorcotinine.

20

11.1. MATERIALS AND METHODS

         Instrumentation included an SLT Lab
instruments 340ATTC Microtiter Plate Reader, COBAS
MIRA, and Varion VXR-200. Urine samples were from a
25   general population previously analyzed for cotinine.
Samples were stored at -20 degrees.
         All chemicals were from Sigma Aldrich unless
otherwise stated. Cis and trans-hydroxycotinine were
purchased from the laboratory of George Neurath (See
30   Neurath et al., supra). Glucose-6-phosphate
dehydrogenase was from Beckman. The Nicotine
Metabolite Assay Kit, NiMA AutoMates™, and the ELISA
Kits, Tobacco Screen®, and the Cotinine Trace
Quantities, CotiTraq®, TMB chromogen system, anti-
35   Cotinine antisera, peroxidase lab lled anti-Cotinine,

and Cotinine urine standards are commercially
available from Serex, Inc. (Maywood, NJ). Preparation
of anti-cotinine antisera is described in Section
6.1.4., <u>Supra</u>.

5

11.1.1. PREPARATION OF RELANDS

1-Isopropyl-4-carboxy-5-(3-pyridyl)-2-
pyrrolidinone, (hereafter, N-isopropyl-4-carboxy-
norcotinine) and 1-propyl-4-carboxy-5-(3-pyridyl)-2-
10 pyrrolidinone (hereafter, N-propyl-4-carboxy-
norcotinine) (Figure 8) were prepared according to the
method of Cushman & Castagnoli (1972, *J.Org. Chem.*
37:1268). Briefly, to a solution of 17 g of pyridine-
3-carboxy-aldehyde in 50 ml of benzene was added a
15 benzene solution of 8 g isopropyl amine (or 8 g propyl
amine) and 12 g molecular sieve pellets. The mixture
was stirred at 20°C overnight in a flask. The
solution was filtered through two layers of Whatman
No. 2 filter paper and evaporated under reduced
20 pressure to give the imine as a yellow oil. The
structure of the products was confirmed by ^{1}H NMR.

N-isopropylcarboxynorcotinine and N-
propylnorcotinine were prepared as follows. Twelve g
of N-3-pyridylidene isopropyl imine or N-3
25 pyridylidene propyl imine and 15 g succinic anhydride
were refluxed for 24 hours in 100 ml xylene. After
the mixture cooled, the top layer was decanted and
discarded. The residue brown oil was dissolved in 300
ml of 5% sodium bicarbonate solution, washed with two
30 250 ml portions of chloroform, and decolorized by
absorption with 1 g activated charcoal. The
suspension was filtered and the yellow filtrate heated
on a steam bath to remove traces of chloroform. The
pH was adjusted to 4.7 with phosphoric acid to
35 precipitate the product. The crude carboxylic acid

was collected by filtration and r crystallized from a
boiling ethanol to give 4 g white crystal. The
structures of the compounds were confirmed by ^{1}H NMR.

5   11.1.2.   PREPARATION OF GLUCOSE-6-PHOSPHATE
                DEHYDROGENASE CONJUGATES

          Conjugates of N-isopropyl-4-carboxy-
norcotinine, N-propyl-4-carboxy-norcotinine and cis
3'-hydroxycotinine to glucose-6-phosphate
10 dehydrogenase were prepared according to the methods
described by Rubenstein and Ullman (1975, U.S. Patent
No. 3,875,011). Briefly, to 1 ml of 0.1 M sodium
carbonate buffer, pH 9.0, were added 0.43 ml of
glucose-6-phosphate dehydrogenase (2.8 mg), 20 mg of
15 NADH (disodium salt), 10 mg of glucose-6-phosphate,
and 300 μl of carbinol. The solution ("enzyme
solution") was stored at 4°C to chill.
          To an empty test tube were added 26 mg of N-
propyl or N-isopropylcarboxynorcotinine, 11.5 mg of N-
20 hydroxysuccinimide, 20.6 mg of dicyclohexyl-
carbodiimide, and 1.0 ml of dimethylformamide. This
mixture was left at room temperature for 1 hour to
allow the activated cotinine ester to form. After 1
hour, 10 μl of the reaction mixture were added to the
25 cold enzyme solution at 15 minute intervals until a
total of 70 μl were added (90 minutes total). Fifteen
minutes after the final addition of reaction mixture,
the modified enzyme was dialyzed against five changes
of 1 liter each of 0.055 M Tris-HCl buffer, pH 7.9,
30 for at least three hours each.

     11.1.3.   REAGENTS FOR THE HOMOGENEOUS RELEASE ASSAY
          Reagent solutions for the homogeneous assay
of cotinine were prepared as three separate solutions,
35 reagents A, A+, and B. Reagent A consisted of
glucose-6-phosphate dehydrogenase conjugate at a

protein concentration of 0.74 μg/ml, 0.05 M Tris
buffer, 5 mM MgCl$_2$, 0.5 mM EDTA, 1.75 mg/ml glucose-6-
phosphate, 0.5% BSA, and preservatives at pH 7.9.
Reagent A+ consisted of antisera in reagent A buffer.

5   Reagents A and A+ were mixed prior to use to form
working solution A, which is stable for one week at
4°C.

Reagent B consisted of NAD at 3.3 mg/ml in
0.02 M Tris buffer, pH 7.0.

10   Cross-reactivity was tested with cotinine
and/or trans-3'-hydroxycotinine solutions prepared as
follows. To 10 ml of a negative urine pool were added
100 μg of cotinine or trans-3'-hydroxycotinine. The
mixture was vortexed and serially diluted into the

15 same negative urine standard to make solutions of 5,
2.5, 1.25, 0.62, 0.31 and 0.16 μg/ml of cotinine or
trans-3'-hydroxcotinine.

To prepare the 1:3, cotinine:trans-3'-
hydroxycotinine, solution, a 10 ml aliquot of negative

20 urine standard was spiked with 100 μg of continine and
300 μg of trans-3'-hydroxycotinine. This solution was
vortexed and serially diluted into the same negative
urine standard to form dilutions of 5 (15), 2.5 (7.5),
1.25 (3.75), 0.62 (1.87). 0.31 (0.94), 0.16 (0.48)

25 μg/ml of cotinine (hydroxycotinine).

Cross-reactivity was calculated using the
following formula;

concentration found (μg/ml)
--- x 100%
concentration of cross reactant

30 (μg/ml) in sample

11.1.4. ASSAY FORMATS

ELISA format. Corning microtiter plates
were coated overnight with 100 μl of either glucose-6-

35 phosphate dehydrogenase conjugated to cis-
hydroxycotine, N-propyl-norcotinine or N-isopropyl-

norcotinine at 1 μg protein per ml of PBS; the wells
were emptied, dried and stored with dessicant until
use. To activate for release, the plate was incubated
<1 hour with 100 μl of peroxidase labelled affinity

5   purified anti-cotinine antibody. Excess antibody was
removed by 2 washes with PBS in 0.05% Tween 20.

       To a microtiter plate coated with a release
ligand-antibody (reland) complex, 10 μl of
urine/standard and 90 μl of distilled water were added

10   to each well. After 2 minutes, 50 μl of the
supernatant were transferred to uncoated wells
containing 100 μl of TMB and incubated for 10 minutes.
The reaction was stopped with 100 ml of 1N H_2SO_4 and
A_{450} was read.

15   Homogeneous Release Assay. The homogeneous
assay of the present invention, was performed in the
AutoMates™ format, utilizing the same enzyme system
and the same reagents as the conventional homogeneous
associative assay, but modified as follows to become a

20   dissociative reaction.

Ab:ligand conjugate + analyte ---> Ab:analyte + ligand
                                              conjugate
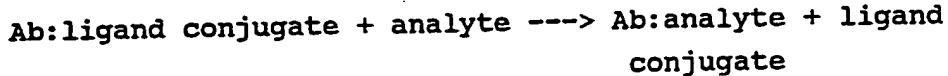
25   Before use, reagent A (enzyme conjugate in
buffer) and reagent A+ (antisera in buffer) were mixed
for a minimum of one hour. The reaction was started
by addition of sample and NAD. As in the associative
assay, enzyme activity was measured by monitoring the

30   formation of NADH at A_{340} nm, and enzyme activity is
directly related to the concentration of analyte in
the sample.

       The homogenous assay of the invention using
AutoMates™ was performed on the COBAS MIRA according

35   t the application sheet parameters. Two hundred μl

of reagent A were incubated with 10 μl of r agent B
(NAD) and the mixture was incubated for 25 seconds.
The absorbance was read over the final 200 seconds.
Total time of the assay was 5.0 minutes. Greater
5 sensitivity of a sample containing 10 ng/ml of analyte
was observed with a 25 μl sample.

Associative Homogeneous Assay. The NiMA
AutoMates™ format is a homogeneous, associative,
competitive assay, like that described by Rubenstein,
10 Schneider, and Ullman (1972, supra). There are two
steps to the immune reaction:

Ab + analyte ---> Ab:analyte + Ab
Ab:analyte + Ab + conjugate --->Ab:conjugate
15   +Ab:analyte
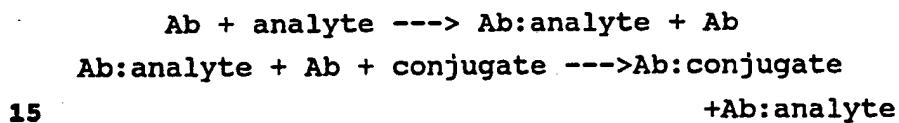
Briefly, sample was pre-incubated with
antisera for several minutes. Into this reaction
mixture was added glucose-6-phosphate dehydrogenase
20 conjugated with cotinine. Antibody that has not
interacted with cotinine in the sample binds to
cotinine on the glucose-6-phosphate dehydrogenase.
The binding of antibody to the enzyme-linked ligand
inhibits enzyme activity, thus the enzyme activity is
25 directly related to the concentration of analyte in
sample. Enzyme activity of glucose-6-phosphate
dehydrogenase was measured by monitoring at A_{340} nm the
formation of NADH, which forms as the enzyme oxidizes
glucose-6-phosphate to glucono-δ-lactone-6-phosphate
30 and reduces NAD to NADH.

NiMA AutoMates™ was performed on the COBAS
MIRA according to the application sheet parameters.
Two hundred μl of reagent A were incubated with 10 μl
of sample at 37 degrees for 75 seconds. Fifty μl of
35 reagent B were added and the mixture was incubated for

25 seconds. The absorbance was read over the final
250 seconds. Total time of the assay was 5.83
minutes.

11.2. RESULTS

11.2.1. HETEROGENEOUS RELEASE ASSAY (ELISA FORMAT)

Release of labelled antibody complexed to
solid phase was detected in supernatant when free
cotinine was present in sample (Figure 9). Unlike
conventional competitive immunoassays, absorbance or
signal was directly proportional to analyte
concentration.

The cis-hydroxycotinine conjugate bound and
released the antibody most efficiently, but had the
highest background.

11.2.2. HOMOGENEOUS FORMAT

Dose response curves for the associative
(NiMA) and release homogeneous assays demonstrate the
greatly increased range of the release assay (Figure
10). Comparison of the associative and release
formats is presented in Table 9.

TABLE 9. COMPARISON OF A HOMOGENEOUS DISSOCIATIVE
ASSAY FOR COTININE AND A HOMOGENEOUS
ASSOCIATIVE ASSAY FOR COTININE (NiMA).

	RELEASE	NiMA
Final Antisera Dilution	4.8×10^{-3}	2.4×10^{-3}
Final Conjugate Concentration	0.51 μg/mL	0.03 μg/mL
Antisera Dilution/μg Conjugate	9.6×10^{-3}	80×10^{-3}
Lower Limit	0.01 μg/mL	0.05 μg/mL
Upper Limit	1000 μg/mL	2 μg/mL
Time	5 min	5.8 min

Table 9 shows that the release assay utilizes 17-fold more enzyme and two-fold more antibody than the associative assay. But the increased enzyme and antibody do not result in

5 decreased sensitivity as they do in associative immunoassays: smaller amounts of all reactants can be utilized in release, but this limits upper range of the assay. Figure 11 shows the range of the assay, 0.01-100 µg/ml. In the example shown, a seventeen-

10 fold increase in reactants yields a greater than 1,000-fold increase in range of the assay, with no loss of sensitivity at the low end of the curve. This formulation is sensitive to 10 ng/ml and can be used for quantitating saliva samples. The antibody to

15 enzyme ratio of the release to associative assays is 10:80; that is the associative assay uses 8-fold more antibody per enzyme molecule than the release. In the release assay all antibody molecules can be bound to ligand conjugated to enzymes and are capable of being

20 released by analyte. In the associative assay there is a large excess of antibody, which decreases reaction time. The ability of the release assay to monitor the activity of a much larger percentage of the antibody in the reaction mixture increases

25 sensitivity and decreases background noise and reaction time.

11.2.3. CROSS-REACTIVITY: TRANS-HYDROXYCOTININE

The specificity of the release assay

30 relative to the associative assay was confirmed by comparing the release and association formats for cross-reactivity with trans-3'-hydroxycotinine (Table 10).

35

TABLE 10. INTERFERENCE OF TRANS-HYDROXYCOTININE
IN RELEASE AND ASSOCIATIVE (NiMA)
HOMOGENEOUS ASSAYS FOR COTININE.

RELEASE ASSAY

SPIKE CONC. (μg/mL)	COTININE SPIKE		TRANS-HYDROXYCOTININE SPIKE	
	CONCENTRATION FOUND (μg/mL)	% RECOVERY	CONCENTRATION FOUND (μg/mL)	% RECOVERY
10	9.03	90	1.69	16.9
5	4.22	84	0.72	14.4
2.5	2.15	86	0.29	11.6
1.25	1.15	92	0	0
0.62	0.63	102	0	0
0.31	0.25	81	0	0
0.16	0.14	88	0	0
AVERAGE		89		6.1

NiMA

SPIKE	COTININE SPIKE		TRANS-HYDROXYCOTININE SPIKE	
	CONCENTRATION FOUND (μg/mL)	% RECOVERY	CONCENTRATION FOUND (μg/mL)	% RECOVERY
10	11.85	119	1.87	18.7
5	5.6	112	0.85	17
2.5	2.03	81	0.44	17.6
1.25	1.59	127	0.21	16.8
0.62	0.59	95	0.17	27.4
0.31	0.49	158	0.11	35.5
0.16	0.13	81	0.08	37.5
AVERAGE		110		24.4

The release assay showed one-fourth the
cross-reactivity with hydroxycotinine of the
associative assay. In the release assay there was no
cross-reactivity at the low end of the curve. The
greatest amount of interference in the associative
assay was seen at the low end of the curve. To
evaluate the actual effect of trans-3'-hydroxy

cotinine on the assay of urine samples, samples were
spiked with cotinine:trans-3'-hydroxycotinine in a 1:3
ratio (this is the ratio reported to exist in smokers'
urine (Engvall et al., 1971, Immunochemistry 8:871))
5   and assayed in both release and associative
homogeneous assays (Table 11).

TABLE 11.   CROSS REACTIVITY OF TRANS-HYDROXCOTININE IN
            THE PRESENCE OF COTININE IN THE RELEASE AND
            THE ASSOCIATIVE (NiMA) HOMOGENEOUS ASSAYS
10   FOR COTININE.

COTININE (TRANS HYDROXYCOTININE) µg/mL	RELEASE		NiMA	
	COTININE CONC. FOUND (µg/mL)	%COTININE RECOVERY	COTININE CONC. FOUND (µg/mL)	% COTININE RECOVERY
10 (30)	16.82	168	>20	>200
5 (15)	7.55	151	14.21	284
2.5 (7.5)	3.69	148	4.38	175
1.25 (3.75)	1.45	116	2.29	183
0.62 (1.9)	0.92	155	1.82	294
0.31 (0.93)	0.4	129	0.54	174
0.16 (0.48)	0.23	144	0.37	231
AVERAGE		144		220

        Trans-3'-hydroxycotinine contributed to
25   cotinine quantitation in both the release and the
associative assays. The contribution is rather
constant across the range of the assay in both formats
but results in an average 220% recovery in the
associative assay but only 144% in the release assay,
30   indicating that the release assay is less subject to
interference by cross-reactivity, therefore reducing
the potential for false positive results in an assay
for cotinine.
        The results for cross-reactivity in Table 10
35   suggest that trans-hydroxycotinine present in a sample

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at the expected 3:1 ratio to cotinine would increase
the apparent detection of cotinine by about 45% (3
times about 15% cotinine recovery). Thus the expected
recovery of cotinine in the release assay reported in
5 Table 11 is about 145%. In fact, the average percent
cotinine recovery was about 144%. A similar analysis
for the associative (NiMA) format indicates that the
same 3:1 trans-hydroxycotinine to cotinine ratio would
increase the apparent cotinine recovery by about 60%
10 (3 x 20%). Thus the expected recovery of cotinine in
the associative (NiMA) assay reported in Table 11 is
160%. However, the average % cotinine recovery is
220%, and ranged as high as 294%. Thus there appears
to be a "synergystic" effect of trans-hydroxycotinine
15 on cotinine detection, so that the presence of trans-
hydroxycotinine skews results in the associative assay
for cotinine in an unexpected, and therefore perhaps
uncorrectable, way.

       The release assay exhibits cross-reactivity
20 as predicted, but the associative format shows average
recoveries of 220%, almost double that predicted by
the simple cross-reactivity data. This demonstrates
that the associative assay is more subject to
interference than release.

25

       11.2.4. CROSS-REACTIVITY OF
                N-ISOPROPYL-4-CARBOXY
                NORCOTININE
       To further test the stability and
releasability of the antibody-ligand complex we
30 characterized the ability of N-isopropyl-4-carboxy-
norcotinine to interfere in the various release
assays, all of which used the same antibody. Results
are shown in Table 12.

35

TABLE 12.   CROSS REACTIVITY FOR N-ISOPROPYL-4-CARBOXY-
             NORCOTININE IN ASSOCIATIVE AND RELEASE
             ASSAYS.

SPIKES URINE(μg/mL)	%CROSS REACTIVITY		
	CotiTraq®	Release	NiMA
0.24		0%	<0
0.5	0%	0%	<0
2	0%	0%	<0
4	0%	0%	0.05%
10	0.3%	0%	0.11%
100	0.3%	0.4%	0.24%

The ligand N-isopropyl-4-carboxy cotinine
showed less than 0.4% cross-reactivity, even in
concentrations as high as 100 μg/ml. The ligand shows
no cross-reactivity with the antibody complexed to it
on G-6-P-DH until 100 μg/ml, at which point cross-
reactivity of 0.4% was detected. N-isopropyl-4-
carboxy-cotinine interfered more in the conventional
NiMA and CotiTraq® Assay format than in the release
assay, indicating that it is not a competitor for the
antibody in the release format.

The release homogeneous assay and
conventional homogeneous and conventional ELISA
formats were also compared. The release heterogeneous
assay, NiMA AutoMates™ and Tobacco Screen® (ELISA)
assays for cotinine were compared using a cotinine
equivalent cutoff of 0.5 μg/ml (0.5μg/ml is considered
equivalent to urine composition of 0.25 μg/ml
cotinine, 0.75 μg/ml hydroxycotinine in Tobacco
Screen® and NiMA, and 0.35 μg/ml cotinine in the
release assay of cotinine). Table 13 shows that
release correlates 100% with Tobacco Screen®. Tobacco

Screen® correlates 100% with HPLC results using an HPLC cutoff of 200 ng/ml and a Tobacco Screen® cutoff of 400 ng/ml.

5   TABLE 13.   COMPARISON OF RELEASE HOMOGENEOUS COTININE
             ASSAYS WITH THE ASSOCIATIVE ASSAYS, TOBACCO
             SCREEN® (ELISA) AND NiMA (HOMOGENEOUS),
             USING 0.5 μg/mL CUTOFF FOR ALL ASSAYS.

TOBACCO SCREEN®   NiMA

10

		+	−			+	−
	+	106	0		+	106	0
Release				Release			
	−	0	34		−	0	34

n = 140   n = 140

15

11.2.5. ASSAY PRECISION

The release and NiMA homogeneous assays for cotinine were evaluated for precision on a COBAS MIRA using negative, 0.5 μg/ml and 2 μg/ml urine samples
20   (Table 14). Precision is used to indicate the coefficient of variation of repetitive tests on the same sample. The release assay showed more than a two-fold improvement in precision over the associative assay. Even though reactant concentrations are 17-
25   fold higher than NiMA, the release assay had better precision.

30

35

TABLE 14.   PRECISION OF THE RELEASE AND THE
             ASSOCIATIVE NiMA HOMOGENEOUS ASSAYS FOR
             COTININE.   RESULTS STATED ARE REACTION RATE
             IN mA/MIN.

RELEASE

Negative Control 0.0 µg/mL	Cutoff Control 0.5 µg/mL	Positive Control 2.0 µg/mL
n = 15	n = 15	n = 15
avg = 177.79	avg = 185.4	avg = 194.56
SD = 0.88	SD = 0.89	SD = 0.81
CV = 0.5%	CV = 0.5%	CV = 0.4%

NiMA

Negative Control 0.0 µg/mL	Cutoff Control 0.5 µg/mL	Potitive Control 2.0 µg/mL
n = 15	n = 15	n = 15
avg= 49.52	avg= 55.86	avg = 58.27
SD = 0.69	SD = 0.75	SD = 0.78
CV = 1.3%	CV = 1.3%	CV = 1.3%

11.3.   DISCUSSION

11.3.1.   HETEROGENEOUS FORMAT

A release ELISA assay well contains less
than 1/5 the antibody utilized in conventional ELISA
assays. Sensitivity is enhanced as a result because
non-specific activity decreases. Sensitivity is also
enhanced by the almost ten-fold higher enzyme activity
of the released peroxidase-labelled antibody.

In the ELISA format this end point release
assay reduces the time for the assay from 1.5 hour to
under 15 minutes (2 minutes for the release reaction
and 10 minutes for TMB color development). Time could
be further shortened, for example, by automating the
assay steps such as by running a rate reaction assay
on an automated instrument. The release assay also
reduces the number of assay steps by at least half. A

further advantage is the release gives a positive
signal in the presence of analyte.

11.3.2. HOMOGENEOUS FORMAT

5   In the homogeneous assay format, sensitivity
is further enhanced by the fact that released
peroxidase- labelled antibody has much higher activity
than enzyme on the plate. That is, if one measures
released peroxidase conjugated antibody and that
10   remaining on the plate, one can see that a 0.1 O.D.
drop in plate absorbance can result in generation of
2.0 O.D. units in the supernatant. This 10- to 20-
fold enhancement of free enzyme activity is probably
in large part due to a decrease in the effects of
15   diffusion on the released enzyme reaction rate.

        The range of the release assay is extended
because the release assay is a system that starts in
equilibrium. It is therefore possible to use higher
starting concentrations of enzyme complex without
20   increasing noise or losing low-end sensitivity. In
conventional immunoassays, addition of more reagents
changes the sensitivity of the assay by shifting final
equilibrium conditions.   Enzyme concentration
demonstrating release assay were 0.50 μg/ml as
25   compared to 0.03 μg/ml for conventional homogeneous
(associative) assays. However, the conventional type
assay had about 8 times as much antibody:enzyme as the
release assay. The use of monoclonals will further
improve the ratio of antibody to enzyme in the release
30   format. The ratio of antibody to enzyme determines
assay sensitivity.

        While other assay systems that involve
dissociation of preformed antibody-ligand complexes
(Cocola et al., 1979, *Analytical Biochem.* **99**:121-8-
35   Hinds et al., 1984, *Chin.Chem.* **30**:1174-8;

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Hinds et al., 1985, *Chin.Chem.Acta* 149:105-15) have utilized competitive ligands as binding partners, the release assay is a non-competitive system. This is demonstrated in Table 12, <u>supra</u>, where release ligand

5   (or reland) cannot compete the antibody off the antibody-ligand complex. It is also noteworthy that unlike the release assay of the present invention, the competitive dissociation assays described by Cocola et al., Hinds et al., 1984 and Hinds et al., 1985, <u>supra</u>,

10 have not shown significant improvement over associative methods of immunoassay. Although the present invention is not bound by any particular theory, we hypothesize that antibody binds to the release ligand via very low affinity interaction, and

15 in the absence of higher affinity binding partners, antibody undergoes a conformational change to a metastable complex that is releasable and has a time stable affinity constant for release. The complex may become too stable, as observed with conventional

20 ligand conjugates. N-isopropyl-norcotinine was designed to provide a bulky group at a non-immunologically critical site to allow dissociation after equilibrium.

        The rapid dissociation of the reland complex

25 in the presence of ligand may be analogous to turn-over in antibody catalysis (Benkovic et al., 1990, *Science* 250:1135-8) in which antibody prepared to a transition state substrate conformation binds a substrate, induces the transition state conformation

30 in the substrate, and then releases the cleaved substrate rapidly since the products no longer appear in the transition state.

35

11.3.3. CROSS-REACTIVITY

The release assay is less subject to cross-reactivity. More importantly, it was not subject to the synergistic type of cross-reactivity that was observed with conventional immunoassay. We have observed this synergistic enhancement of interference in other conventional immunoassay formats. This may be a generalized phenomenon and perhaps all cross-reactivity ought to be reported as % cross-reactivity seen in the presence of analyte at 50% binding, so that the true contribution to the assay can be assessed.

11.3.4. ASSAY PRECISION

The two-fold improvement in precision seen with the release assay of the invention is probably multifactorial: the starting system is in equilibrium and only one reaction -- dissociation -- occurs, and the matrix, as evidenced by lowered cross-reactivity, probably has less effect on the reaction.

11.4. CONCLUSION

We have developed an assay method utilizing the ability of antibodies to assume an induced fit with a binding partner for which it has very low affinity, the release ligand, or reland. The release assay provides a preformed receptor-reland complex, which can be rapidly dissociated in the presence of analyte. The release system can be used in all immunoassay formats. The release assay has inherent advantages over conventional or associative assays:

1. By eliminating one step in the immune reaction, release saves time and steps and possible sources of error, thereby shortening assay time and simplifying assay t chniques.

2.   Release, i.e., dissociation, is inherently less
     subject to interference making it more accurate.

3.   The ability to monitor all antibody in the assay
     reduces noise and makes a 1000-10,000-fold
5   sensitivity range possible. This methodology,
     using more sensitive markers, extends the
     theoretical range both up and down from that
     available in conventional assay formats.
     Addition of more reactants does not lower
10   sensitivity as in conventional immunoassays, but
     extends the upper range of sensitivity.

4.   An important advantage of release is the mild
     conditions under which the dissociation occurs.
     This allows the solid phase or complex to be
15   regenerated. This should advance the
     possibilities for biosensors. A major problem
     with biosensors is that the solid phase is
     usually a disposable; with the release assay the
     solid phase can be continually regenerated.

20 5. The large range, the positive correlation with
     presence of analyte, and the low noise of the
     system indicates that the release assay format
     can be used to screen for many analytes in one
     reaction mixture.

25

            12.   EXAMPLE: RELEASE ASSAY FOR
                  A COCAINE METABOLITE

         Benzoylecgonine is a metabolite of cocaine.
     Release immunoassays that allow detection of soluble
     benzoylecgonine in a sample are described below. In
30   particular, release of an enzyme-labeled anti-
     benzoylecgonine antibody can be detected in assay
     solution supernatant when free benzoylecgonine is
     present. The following example demonstrates an
     effective release assay for benzoylecgonine. Two
35   assay parameters, the effect of the relative amount of

antibody-label conjugate incubated on a ligand-protein
coated plate and incubation time after addition of
sample on detection of released label, were
investigated.

5

12.1. MATERIALS AND METHODS

Using standard techniques, ecgonine was
conjugated to BGG via a bulky linker (p-aminobenzoic
acid) . Anti-benzoylecgonine-peroxidase conjugate was
10 prepared using standard techniques.

12.1.1. TITRATION OF CONJUGATE

Microtitre plates were coated with
benzoylecgonine-BGG ligand. After washing, 100 μl of
15 antibody-peroxidase conjugate were added to each well
at either 1:500 or 1:1000 dilution and incubated at
r.t. for 60 min and washed twice with wash buffer.
Benzoylecgonine (BE) standard solutions (100 μl) were
added to individual wells. Benzoylecgonine was
20 present at the following concentrations in distilled
water or urine: 0, 0.025, 0.3, and 5.0 μl/ml. After
adding sample, plates were incubated 10 min with
shaking at r.t., and 100 μl of supernatant were added
to replicate wells of an uncoated microtitre plate.
25 To each well of assay supernatant were added 100 μl of
2x TMB substrate solution. The plates were incubated
6 min at r.t., and the reaction stopped by addition of
50 μl of 2 N H_2SO_4 to each well. Absorbance at 450 nm
was measured in a plate reader.

30

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12.1.2.   ASSAY FOR INFLUENCE OF INCUBATION
TIME AND MATRIX COMPOSITION

The procedure described above (Section
12.1.1.) was followed. To detect the effect of matrix
5   composition, standards in a synthetic urine matrix
were diluted in deionized water. To assay the effect
of incubation time, i.e., time needed to detect
release of labeled antibody, peroxidase labeled anti-
benzoylecgonine ecgonine-BGG-coated plates were
10 incubated 0, 2 and 10 minutes after addition of free
benzoylecgonine before transfer of supernatant to a
clean microtiter plate.

12.2.   RESULTS

15   Free benzoylecgonine in sample resulted in
release of antibody-peroxidase conjugate. The amount
of conjugate released correlated with the amount of
conjugate added to the benzoylecgonine-BGG coated
plate as well as with the amount of analyte present in
20 the sample (Table 15). Thus more conjugate is
released from incubation with a 1:500 than a 1:1000
diluted solution.

25

30

35

TABLE 15. RELEASE OF ANTI-BENZOYLECGONINE-PEROXIDASE
FROM ECGONINE-PABA-BGG-COATED PLATE.

Ecgonine conc. (μg/ml)	A_{450} of Released Anticotinine-Peroxidase	
Stds in Water	1:500	1:1000
0	0.881	0.574
0.025	1.541	0.944
0.30	2.465	1.630
5.0	2.859	2.218
Stds in Urine		
0	0.234	0.112
0.025	0.493	0.255
0.30	1.182	0.630
5.0	1.901	1.010

Matrix effects are also evident from the
data in Table 15. In particular, release appears to
be more effective in water than in urine. These
results are expected since matrix is known to affect
antibody antigen reactions. Nevertheless, the
relative release of antibody-enzyme conjugate is not
affected by matrix composition. This relationship can
be seen by comparing the ratio of A_{450} when free
benzoylecgonine is present to A_{450} blank in each matrix
(Figure 12). Thus, the matrix effect appears to
result from inhibition of enzyme by components in
urine and does not appear to affect the amount of
antibody-enzyme conjugate released.

The time of incubation appears to have
minimal effect on enzyme activity in supernatant as
shown graphically (Figure 13).

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12.3. <u>DISCUSSION</u>

Thes results indicate that release of
labeled antibody from a ligand coated plate is
proportional to the amount of labeled antibody added
5   to the plate.

The results also indicate that while sample
matrix affects the absolute enzyme label activity in
release supernatant, this effect can be corrected for
by normalization over baseline control activity.
10 Generally, normalization of various samples allows
comparison and quantification of free ligand, in this
case free benzoylecgonine, present in the sample.

Finally, the results in Figure 13 indicate
that release is nearly complete almost immediately
15 after addition of free ligand, demonstrating that
release assays may be run in short period of time.

13. EXAMPLE: RELEASE ASSAY ON
ANTIBODY-COATED MEMBRANES

In a particular embodiment, presence of
20 soluble analyte may be detected by release of labeled
ligand from an antibody-coated solid phase support,
e.g., a membrane. This example demonstrates release
of labeled ecgonine from a complex with solid phase
25 antibody specific for benzoylecgonine.

13.1. <u>MATERIALS AND METHOD</u>
13.1.1. <u>LABELED LIGAND</u>

Ecgonine was linked to alkaline phosphatase
30 via a spacer linker. In 20 ml of DMSO (Sigma) were
dissolved 40 mg ecgonine (Alltech), 32 mg
N-hydroxysuccinimide (Sigma) and 56 mg of 1-ethyl-
(3-aminopropyl)carbodiimide (Pierce) at r.t. for
4 hrs. A solution of 25 mg N-(4-aminobenzyl)-6-
35 aminocaproic acid (Aldrich) in 0.5 ml DMSO was added
to 2.0 ml of 0.2 M sodium bicarbonate, pH 7.8, and

this mixture was added to the activated ecgonine
solution. The resulting solution was mixed for 1 hr.
at r.t. and held at 4°C overnight. To 1.0 ml of
activated ecgonine-linker solution were added 0.3 ml
5   of 10 mg/ml N-hydroxysuccinimide and 16.7 mg/ml 1-
ethyl-3-(3-dimethylamino)carbodiimide (Sigma) in DMSO,
and the resulting mixture shaken for 4 hrs at r.t. To
1 ml of a solution of 1 mg/ml alkaline phosphatase
(Biozyme ALPI IIG, 1650 U/mg) were added 0.65 ml of
10 activated ecgonine-linker, and the mixture incubated 2
hrs at r.t., then held at 4°C overnight. The product
was dialyzed in the cold against 0.5 M carbonate
buffer, pH 7.8, for 8 hours, with dialysis fluid
changed every 4 hours.
15

13.1.2. ANTIBODY-COATED LATEX MEMBRANE ASSAYS

A suspension of 0.5 ml of polystyrene latex
particles, 0.776 microns (IDC) mixed with 3.5 ml of
phosphate buffer pH 7.5 was prepared. Goat anti-
20 rabbit IgG, Fc fraction, was diluted to 0.5 mg/ml in
phosphate buffer (0.2 ml to 4 ml). The two were mixed
by inversion and incubated at room temperature for 1
hour, and then stored at 4°C. 1 ml of this latex
preparation was centrifuged for 5 minutes at 3000 RPM,
25 washed with 0.01 M phosphate buffer, pH 7.5, and
recentrifuged. The latex particles were resuspended
in 5 ml of phosphate buffer containing 0.1% Tween 20.
55 μl of rabbit antibody against benzoylecgonine were
diluted in 5 ml of the Tween/phosphate and added to
30 the latex suspension. The mixture was gently mixed at
room temperature for 60 minutes. It was then
centrifuged for 5 minutes at 3000 RPM, washed twice
with Tween/phosphate with centrifugation and
resuspension. The preparation was resuspended in
35 Tw en/phosphate buffer containing 0.1% sodium azide.

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Membranes were prepared by soaking 0.45 μ filtration membranes in 80 μl of Tween/phosphate buffer, and 20 μl of anti-benzyolecgonine-coated latex were added and allowed to absorb. Then 10 μl of
5   labeled ecgonine were added to each latex spot. The membranes were incubated 3 hrs at r.t. to allow stable receptor-ligand complex to form and subsequently washed three times with phosphate buffer containing 0.1% Tween 20. Sample or standard was added (100 μl),
10   and the membrane incubated 10 min. at r.t. with shaking. The test was generally run as a visual test by adding substrate directly to membranes where it is washed away released ligand-enzyme samples. Decreased color-intensity was proportioned to the
15   benzoylecgonine concentration in the sample.

For quantitating release the liquid phase (supernatant) was collected by filtration and 50 μl aliquots were added to wells of microtiter plates. To each well were added 100 μl of p-nitrophenylphosphate
20   (Sigma) in diethanolamine buffer, pH 9.7, and the plates were incubated 30 min at room temperature. Absorbance at 405 nm was measured.

13.2.   RESULTS

25   The results of this assay are shown in Table 16.

TABLE 16. Enzyme Activity of Liquid Phase

BENZOYLECGONINE CONC. (μg/ml)	A_{405}
0	0.214
5	0.352

These results demonstrate release of
35   ecgonine-linker-alkaline phosphatase from the latex-

antibody pr paration when free benzoylecgonine is present in sample.

14. EXAMPLE: RELEASE ASSAY FOR TETRAHYDROCANNABINOL (THC)

A solid phase membrane assay, similar to that described in Section 13, supra, has been prepared to allow detection of tetrahydrocannabinol, the major active component of marijuana.

14.1. MATERIALS AND METHODS

14.1.1. PREPARATION OF MEMBRANES

Polyclonal antibodies (sheep) to THC were used. Ammonium sulfate purified antibody, 13 mg/ml in 0.5 M carbonate buffer pH 9.3 in volume of 3 μl, were absorbed to 5 mm disks of Gelman KV3000 ultrabind membranes. The disks were allowed to dry for 30 minutes at room temperature, and then were treated with 0.1% (w/v) bovine serum albumin in carbonate buffer for 15 minutes at room temperature. Excess albumin was removed by aspiration, and membranes were washed for 10 minutes at room temperature with a buffer consisting of 2% (w/v) glucose and 0.01% (w/v) 2,6-di-tert butyl-4-methylphenol (BHT) (Aldrich) in 0.5 M carbonate buffer, pH 9.3. Disks were allowed to dry at room temperature and stored at 4°C.

14.1.2. PREPARATION OF LIGAND

Two mg of 9-carboxy-11-nor-delta-9-tetrahydrocannabinol (C-THC) were dissolved in 0.2 ml of dimethylsulfoxide, and 0.74 mg of n-hydroxysuccinimide were added. After reaction at room temperature for 2 hours, 0.5 ml of this mixture were added to 5 mg of alkaline phosphatase in 3 ml of phosphate buffered saline (PBS). The entire reaction mixture was mixed overnight at room t mperature.

14.1.3. ASSAY PROCEDURE

Disks containing 3 μl of anti-THC antibody
at 1:10 dilution were placed in wells of microtiter
plates. To these disks were added 100 μl of the THC-
5 alkaline phosphatase ligand at a 1:2000 dilution.
Plates were incubated for 30 minutes at room
temperature. To assess the effect of various
components on the stabilization of dried receptor-
ligand complex, various formulations were used to wash
10 the complex prior to drying. Wells were washed with
Tris buffer containing 0.1% Tween 20 or with Tris
buffer containing glucose. The disks were then
allowed to dry and 200 μl of standards containing 0 or
10 μg/ml THC were added to the disks in wells. The
15 assay could then be read visually by adding substrate
to the membrane and noting a definite fading of color
when THC effected release. The reaction could also be
quantitated as described further. The plates were
incubated for 30 min at room temperature and 100 μl of
20 supernatant from each well were transferred to wells
in a clean replicate microtiter plate.

To each well of the replicate microtiter
plate were added 100 μl of p-nitrophenylphosphate
(Sigma). The plates were incubated for 15 minutes at
25 room temperature, and absorbance read at 405 nm.

The remaining incubation mixture of solid
phase antibody and conjugate was incubated for 30 min
at 37°C and 100 μl of this supernatant was transferred
to replicate wells in clean microtiter plates. To
30 each well were added 100 μl of p-nitrophenylphosphate
and the plates were incubated for 15 min at room
temperature, and absorbance measured at 405 nm.

35

14.2. RESULTS

The results of this assay are shown in Table 17.

5   TABLE 17.   RELEASE OF LABELED THC-ALKALINE
PHOSPHATASE CONJUGATE AS A FUNCTION
OF STABILIZATION OF RELEASE COMPLEX.

	30' INCUBATION ROOM TEMP		30' INCUBATION, ROOM TEMP. PLUS 30' AT 37°C	
SAMPLE	TRIS/TWEEN WASH	TRIS/TWEEN/ GLUCOSE WASH	TRIS/TWEEN WASH	TRIS/TWEEN GLUCOSE WASH
0 µg/ml THC	0.101	0.111	0.129	0.212
10 µg/ml THC	0.134	0.152	0.260	0.484

These results show the optimization of incubation times and formulation of washing buffer on the release of THC:alkaline phosphatase conjugate from antibody bound to membranes. In particular, the presence of glucose in the washing buffer enhances released enzyme activity in the supernatant. Most importantly, these results indicate preference of the antibody for the analyte in the sample, thus demonstrating an effective release assay for THC.

It should be noted that the release reaction visualized on membranes was much more dramatic.

15. EXAMPLE: RELEASE ASSAY FOR BETA-BLOCKERS

A homogeneous release assay for beta-blockers is presented. Detection of beta-blockers is useful for monitoring patients on these drugs. In particular, the stability of antibody complexes with ligand was tested.

15.1.   MATERIALS AND METHODS
15.1.1.   LIGAND: ANALYTE-ENZYME CONJUGATE

Atenolol acid (6.5 mg) (Imperial Chemical
Industries) was dissolved in 0.125 ml of 0.1 M sodium
5   carbonate with the aid of 0.015 ml of 1 N HCl. After
dissolution, 0.1 ml of deionized water and 0.25 ml of
dimethylsulfoxide were added, followed by 6 mg of N-
hydroxysuccinimide and 10 mg of EDC. The reaction
mixture was kept at room temperature for 2 hours. To
10   a glass tube were added 1 ml of 0.1 sodium carbonate
buffer, pH 9.0, and 2.8 mg of glucose-6-phosphate
dehydrogenase; after dissolving, 20 mg of reduced
nicotine adenine dinucleotide (NADH), 10 mg of
glucose-6-phosphate and 0.3 ml of carbitol [2-
15   ethoxyethoxy) ethanol] were added. This mixture was
kept at 4°C for 1 hour.

One hundred forty μL of the activated
atenolol were added to the glucose-6-phosphate
dehydrogenase reaction vessel over a period of 90
20   minutes, in aliquots of 10 μl. Fifteen minutes after
the last addition the material was transferred to a
dialysis bag and dialyzed overnight in the cold
against 2 1 of 0.05 M Tris-HCl, pH 7.9. The next day
dialysis was continued for 8 hours with three changes
25   of fluid.

15.1.2.   ASSAY SYSTEM

Reagent A (160 μl) was prepared with 1 μl of
antiserum (rabbit) against 4-hydroxypropranolol, and
30   12 μl of 0.11M glucose-6-phosphate in 0.05 M Tris
buffer, pH 7.9, 3.3 mM magnesium chloride.

Reagent B (30 μl) was prepared with 10 μl of
conjugate of 1:500 dilution, and 8 μl of 0.1 M
nicotine adenine dinucleotide in 0.05 M Tris buffer,
35   pH 7.9.

A and B were mixed for 10 seconds and 10 μl
of sample or standard were added, followed by mixing
for 10 seconds. The mixture was incubated for the
indicated times at 37°C and absorbance at 340 nm
5   measured.

15.2. RESULTS

15.2.1. STABILITY OF ANTIBODY-CONJUGATE COMPLEX

These experiments were designed to determine
10 the stability of the antibody/beta-blocker-enzyme
complex.

The complex was made up with 1.41 ml of
0.05 M Tris-HCl, pH 7.8, 10 μl of undiluted antibody,
80 μl of 0.1 M NAD and, and 100 μl of conjugate at 2
15 mg/ml and kept at room temperature for the indicated
time periods. At specified times, samples containing
free 4-hydroxpropanolol were added to aliquots of the
complex and the mixture was incubated for 5 min at
room temperature. The reagent containing glucose-6-
20 phosphate was then added, and absorbance at 340 nm
measure over a 20 minute time period.

25

30

35

TABLE 18. CHANGE IN OD AFTER DIFFERENT INCUBATION
TIMES OF THE RECEPTOR-LIGAND COMPLEX[1].

4-HYDROXY-PROPRANOLOL SAMPLE	(mA/min)				
	1'	10'	30'	60'	120'
0.1 µg/ml	3.918	2.256	2.083	1.034	4.197
1.0 µg/ml	7.913	4.456	5.427	3.171	6.243
10 µg/ml	12.875	12.855	13.720	10.210	12.759
SLOPE [2]	1.097	1.619	1.425	1.555	1.540
INTERCEPT [2]	-0.120	-0.580	-0.433	09.533	-0.522

[1]   In this assay the release time was 5 min. and
      measurement time was 20 min.

[2]   Slope and intercept were calculated according to
      the least squares method.

        The similarity of the reaction statistics in
Table 18 indicates that the antibody/ligand-enzyme
complex was stable for at least 2 hours at room
temperature, and that effective release could occur in
the presence of free analyte during that time.

    15.2.2.   OPTIMIZING RELEASE TIME AND MEASUREMENT TIME
        In addition to showing that the
antibody/ligand-enzyme conjugate was stable for at
least two hours, the assay was optimized for release
time, i.e., the time after adding sample and NAD
substrate before measuring the change in OD, and
optimal measurement time, i.e., the time from start to
finish for measuring the change in OD. The results of
these optimization assays are shown in Tables 19 and
20.

TABLE 19. <u>OPTIMIZING RELEASE TIME[1]</u>.

4-HYDROXY-PROPANOLOL CONCENTRATION	mOD/MIN AFTER		
	1 MINUTE	3 MINUTES	5 MINUTES
0.1 ug/ml	1.167	0.859	2.895
1.0	3.928	11.382	6.007
10.0	18.334	18.816	9.852
SLOPE[2] INTERCEPT[2]	1.743 -0.665	0.857 0.213	1.0947 -0.117

[1]   Antibody/ligand-enzyme complexes were incubated 10 sec. prior to adding sample; mOD/MIN (rate of absorbance change) was measured for 10 min.

[2]   Slope and intercept were calculated according to the least squares method.

TABLE 20. <u>OPTIMIZING READING TIME[1]</u>.

4-HYDROXY-PROPANOLOL CONCENTRATION	mOD/MIN AFTER		
	7 MINUTE	10 MINUTES	20 MINUTES
0.1 ug/ml	0.476	6.141	3.099
1.0	2.059	14.347	11.364
10.0	6.485	24.554	22.533
SLOPE[2] INTERCEPT[2]	1.481 -0.4771	1.0978 -0.1203	1.1362 -0.1633

[1]   Antibody/ligand-enzyme complexes were incubated 10 min prior to addition of sample; the release time in this assay was 5 min.

[2]   Slope and intercept were calculated according to the least squares method.

Under the condition of this assay, a release
time of 5 min and a measurement time of NAD reduction
for 10 min provide optimum results, with the best
slope and intercept values.

16.   EXAMPLE: HOMOGENEOUS RELEASE ASSAY FOR THIAZIDES
          Thiazides are a class of diuretics, the most
well known of which is hydrochlorothiazide. Detection
of thiazides is important for medical treatment,
particularly in emergency situations.

16.1.   MATERIALS AND METHODS
16.1.1.   LIGAND: THIAZIDE-GLUCOSE-6-PHOSPHATE CONJUGATE

          The ligand-enzyme conjugate was prepared as
follows: 15 mg of hydrochlorothiazide were dissolved
in 0.5 ml of 5 N sodium hydroxide, and placed in
boiling water for 30 minutes. The solution was cooled
to room temperature and acidified with 1 ml of 6 N
HCl. The tube was placed into an ice bath and 0.5 ml
of 0.2 M sodium nitrite added. The mixture was
allowed to remain at 0°C for 10 minutes, and then 0.5
ml of 7% ammonium sulfate were added.

          The pH of the mixture was raised to between
5 and 6 using 100 μl of 0.5 M sodium carbonate. In an
ice bath 280 μl of the mixture were mixed immediately
with 2.8 mg of glucose-6-phosphate dehydrogenase in
1 ml of 0.1 M carbonate buffer, pH 9.0, containing
20 mg of NADH, 10 mg of glucose-6-phosphate and 300 μl
of carbinol, and allowed to remain at 0°C for 30
minutes. The reaction mixture was then dialyzed
against 0.05 M Tris buffer, pH 7.8, for 24 hours at
2-8°C with four changes of buffer.

16.1.2. ASSAY

Reagent A (160 µl) was anti-thiazide antibody (rabbit), 1 µl, in 147 µl Tris-HCl, pH 7.8, with 12 µl of 0.11 M glucose-6-phosphate. Reagent B,

5 (22 µl) was 10 µl of conjugate at 1:250 dilution in 12 µl of Tris-HCl buffer, pH 7.8, containing 3 mM magnesium. Reagents A and B were mixed and incubated for 5 minutes at room temperature. Samples were added in volumes of 10 µl. Incubation was continued at room

10 temperature for 3 minutes, and then 8 µl of 0.1 M NAD were added. Absorbance at 340 nm was measured for a 10 min period.

16.2. RESULTS

15 Free hydrochlorothiazide in sample caused release of antibody from the ligand-enzyme conjugate, resulting in a dramatic shift in the rate of absorbance change. These results are summarized in Table 21.

20

TABLE 21. THIAZIDE RELEASE ASSAY.

HYDROCHLOROTHIAZIDE CONCENTRATION (µg/ml)	RATE OF ABSORBANCE CHANGE (mAU/MIN)
0.1	0.030
1.0	9.501
10	15.399

17. EXAMPLE: IDENTIFICATION OF A LOW AFFINITY ANALOG OF CONTININE BY ITS ABILITY TO COMPETE

30 The second principal urinary metabolite of nicotine, trans-3-hydroxycotinine, was tested for its ability to compete in the enzyme immunoassay for cotinine, which utilizes an antibody raised against

35 carboxycotinine.

17.1. MATERIALS AND METHODS

Trans-hydroxycotinine or cotinine were added
to a synthetic urine matrix in levels from 0.1 to 5
µg/ml, and assayed in an ELISA for cotinine a mixture
5   of cotinine and trans-hydroxycotinine in a 1:3 ratio
were also assayed.

17.2. RESULTS

Trans-hydroxycotine was only 10% as
10 effective as cotinine at inhibiting binding in a
cotinine ELISA. The enhanced binding (greater
absolute OD values) in the presence of hydroxycotinine
is an effect seen before in inhibition assays with low
affinity ligands.
15   The ineffectiveness of hydroxycotinine as an
inhibitor can also be seen in the inhibition curve of
a 1:3 ratio of cotinine to hydroxycotinine. Although
present in 3-fold excess, the hydroxycotinine cotinine
combination is less inhibitory than cotinine alone.
20 There is no observable additive inhibition by
hydroxycotinine.

17.3. DISCUSSION

The cotinine metabolite trans-hydroxycoti-
25 nine; which differs from cotinine only by the presence
of a hydroxyl group at C-3' in transorientation, binds
antibody to cotinine with much lower affinity than
cotinine. This is the characteristic necessary for a
useful release ligand, and in fact, ligands prepared
30 with trans-hydroxycotinine are useful in assays for
cotinine (see Sections 6.-11., supra).
The phenomenon of enhanced binding, i.e.,
greater absolute O.D. values, in the presence of a low
affinity analog is not well understood. It may be due
35 to inhibition of cross-reactive antibodies.

Nevertheless, enhancement of binding in the presence of low concentrations of a potential inhibition indicates useful low affinity ligand for release assays.

5

18. USE OF LOW AFFINITY CHROMATOGRAPHY FOR ISOLATION OF ANTIBODIES

Use of low affinity ligands for chromatography to purify antisera is advantageous

10 because elution under mild conditions yields higher antibody recovery. Such antibodies find particular use in release assays.

Isolation of antibodies to human chorionic gonadotropin (hCG) was accomplished using a column to

15 which a crude extract of sheep gonadotropic hormones had been covalently bound. While these sheep hormones are not biologically active in humans, nor do they crossreact with anti-hCG, there remains enough sequence conservation, especially with sheep

20 luteinizing hormone (sLH), to be able interact with antibodies to hCG under controlled conditions.

18.1. MATERIALS AND METHODS

18.1.1. PREPARATION OF AFFINITY COLUMN

25 A crude extract of sheep pituitary gonadotropins was attached to sepharose using 1 g of cyanogen bromide activated sepharose 4B. The gel was placed in 1.6 ml of 0.1 M carbonate buffer, pH 8.0, containing 0.5 M sodium chloride. 24 mg of the crude

30 extract were added and the material mixed for 3 hours at room temperature. The gel was centrifuged, supernatant discarded, and the gel resuspended in 0.01 M Tris buffer, pH 8.0, and kept at 4°C overnight.

The gel was centrifuged again, supernatant

35 discarded, and the gel suspended in 10 ml of 0.01 M

Tris Buff r, pH 8.0, containing 0.2 M glutamic acid.
This suspension was incubated for 1 hour with mixing.
Then the gel was packed into a 1 x 10 cm column and
washed sequentially with 0.1 M acetate buffer, pH 4.0,

5 0.5 M sodium chloride, and 0.1 M sodium carbonate
buffer, pH 8.0, containing 0.5 M sodium chloride. The
column was then equilibrated with phosphate buffer, pH
7.0, containing 0.5 M sodium chloride and 0.01% sodium
azide.

10

18.1.2. AFFINITY CHROMATOGRAPHY

The column was primed with 1 ml of normal
rabbit serum overnight at 4°C the serum was eluted
with phosphate buffered saline (PBS) followed by

15 rinses with the buffers of pH 4 and 8. 1 ml of rabbit
antiserum to hCG with a titer of 1:100,000 was applied
to the column and allowed to sit 10 min. The column
was sequentially eluted with PBS, pH 4 buffer and pH 8
buffer. Protein content of collected fractions was

20 determined by the Lowry method, and anti-hCG activity
by a solid phase competitive enzyme immunoassay.

18.2. RESULTS

The chromatogram of protein content and

25 antibody activity is shown in Figure 14. The bulk of
the protein eluted in the void volume of the column,
while the majority of the antibody activity eluted
just after the void volume in protein-poor fractions.
A smaller protein peak eluted in pH 4 buffer, no

30 protein eluted in pH 8 buffer.

The titer of the pooled fractions of
antiserum was 1:91,200 and the protein content 2.7 mg,
showing an activity recovery of 91%, far higher than
that reported previously in the literature for

35 affinity-purified antibodies. The specific activity

of the starting antiserum was 2200 U/mg, while that of the pooled purified fractions was 37,000 U/mg, a 17-fold purification.

In order to show that the recovery of antibody by this method was not due to the properties of the Sepharose itself, a column of Sepharose 4B was prepared and normal rabbit serum and antiserum applied and eluted as described previously. All antibody activity eluted in the void volume with the protein; none was retained on the column to be eluted afterward.

The antisera used in the experiment showed a 10% cross-reactivity with sLH after the affinity chromatography this level of cross-reactivity did not change.

18.3. DISCUSSION

The purification of antibodies using low affinity analogs attached to affinity chromatography gels appears to permit efficient recovery of antibodies without denaturation and with enhanced specific activity for antigen.

Affinity purification schemes also provide a method to identify a low affinity analog by its ability to retard elution of specific antibody in a mild eluent. According to the above example, in a release assay for hCG, sheep gonadotropins may serve as ligands.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such

m dificati ns ar intended to fall within the scope of
the appended claims.

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WHAT IS CLAIMED IS:

1. A method for the detection of an analyte in a sample comprising:

     (a) contacting the test sample with a receptor-ligand complex, and

     (b) detecting dissociation of the receptor-ligand complex and binding of receptor with analyte which positively correlates with the presence of the analyte in the sample.

2. A method for the detection of an analyte in a sample comprising:

     (a) forming a receptor-ligand complex,

     (b) contacting the sample with the complex, and

     (c) detecting dissociation of the receptor-ligand complex and binding of receptor with analyte which positively correlates with the presence of analyte in the sample.

3. The method of claim 1 or 2 wherein detecting dissociation is by means of measuring a signal from a label associated with either receptor or ligand.

4. The method of claim 3 wherein the label is selected from the group consisting of an enzyme, a fluorophore, a chromophore, a latex particle, a chemiluminescent agent, radioisotope, a chelating complex, a dye, and colloidal gold.

5. The method of claim 1 or 2 wherein the analyte is cotinine, benzoylecgonine, tetrahydrocanna-binol, beta-blockers, or thiazides.

6. The method of claim 1 or 2 wherein the receptor is a nucleic acid, an antibody, antibody fragment, cell surface antibody receptor, or cell surface receptor fragment.

5

7. The method of claim 6 wherein the receptor is an antibody purified by low affinity chromatograph on a column conjugated with the ligand.

10   8. The method of claim 1 or 2 in which the ligand is selected from the group consisting of an analyte analog, derivative, modification or isomer.

9. The method of claim 1 or 2 in which the
15 ligand or receptor is irreversibly absorbed to a solid phase support.

10. A kit for detecting the presence of an analyte in a sample comprising a preformed receptor-
20 ligand complex and means for detecting dissociation of the complex and binding of receptor with analyte in the presence of analyte in the sample.

11. A kit for detecting the presence of an
25 analyte in a sample comprising receptor and ligand for forming a receptor-ligand complex, and means for detecting dissociation of the complex and release of receptor which positively correlates with the presence of analyte in the sample.

30

12. A kit of claim 10 or 11 wherein the ligand or receptor is irreversibly absorbed to a solid support and the detection means comprises colored latex beads.

35

13. The kit of claim 10 or 11 wherein the analyte is cotinine, benzoylecgonine, tetrahydrocanna-binol, beta-blockers, or thiazides.

5   14. The kit of claim 10 or 11 wherein the receptor is a nucleic acid, an antibody, antibody fragment, cell surface antibody receptor, or cell surface receptor fragment.

10   15. The kit of claim 14 wherein the receptor is an antibody purified by low affinity chromatography on a column conjugated with the ligand.

16. The kit of claim 10 or 11 in which the
15 ligand is selected from the group consisting of an analyte analog, derivative, modification or isomer.

17. The kit of claim 10 or 11 wherein the ligand or receptor is irreversibly absorbed to a solid
20 phase support.

18. The kit of claim 10 or 11 wherein detecting dissociation is by means of measuring a signal from a label associated with either receptor or
25 ligand.

19. The kit of claim 10 or 11 wherein the label is selected from the group consisting of an enzyme, a fluorophore, a chromophore, a latex
30 particle, a chemiluminescent agent, a radioisotope, a chelating complex, a dye, and colloidal gold.

20. A cotinine derivative having an aliphatic chain of three or more carbon atoms at the 1N
35 position.

21. The cotinine derivative of claim 20 in which the aliphatic chain is a propyl group.

22. The cotinine derivative of claim 20 in which
5 the aliphatic chain is an isopropyl group.

23. The cotinine derivative of claim 20 conjugated to an enzyme.

10   24. The cotinine derivative of claim 23 in which the enzyme is glucose-6-phosphate dehydrogenase.

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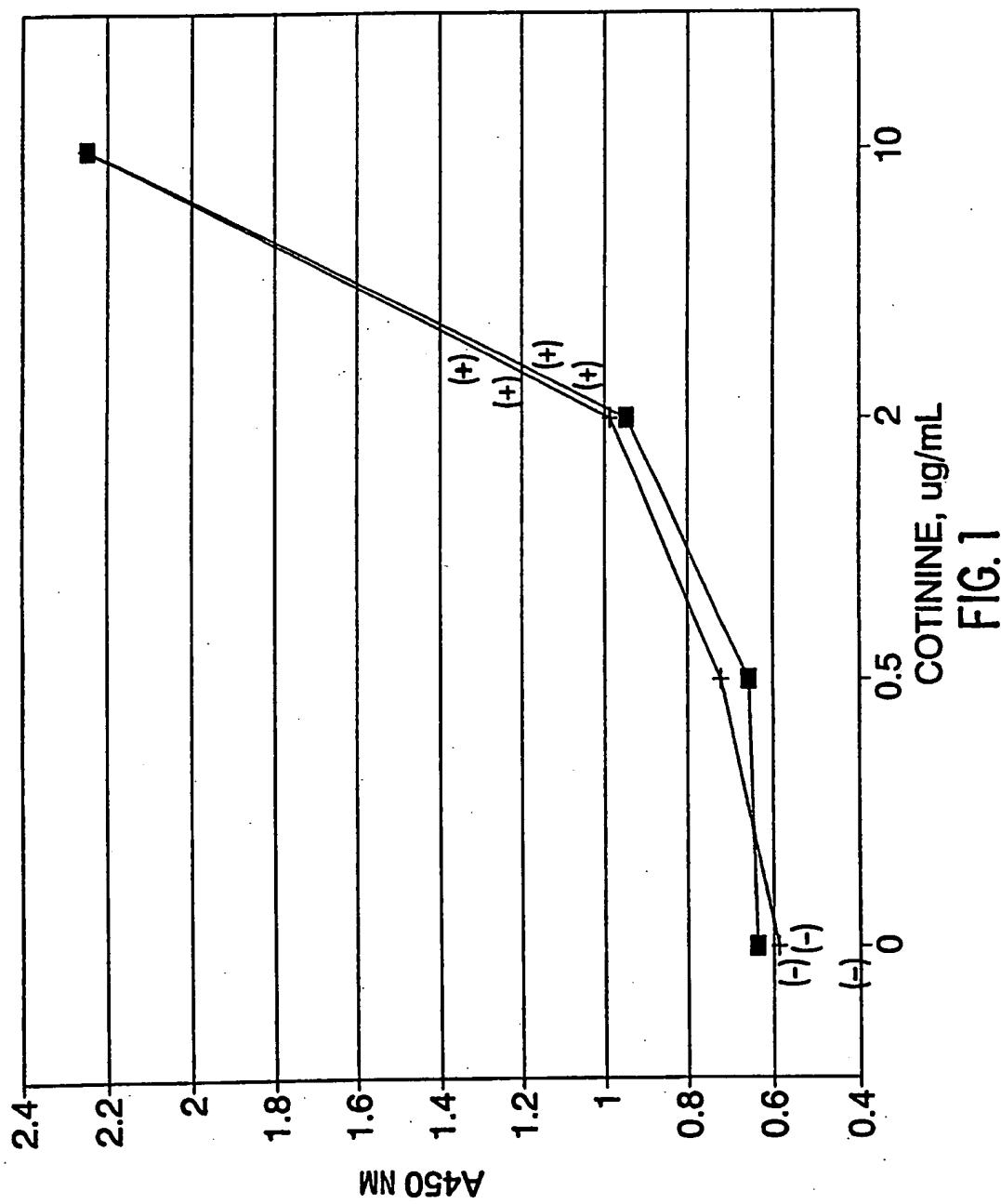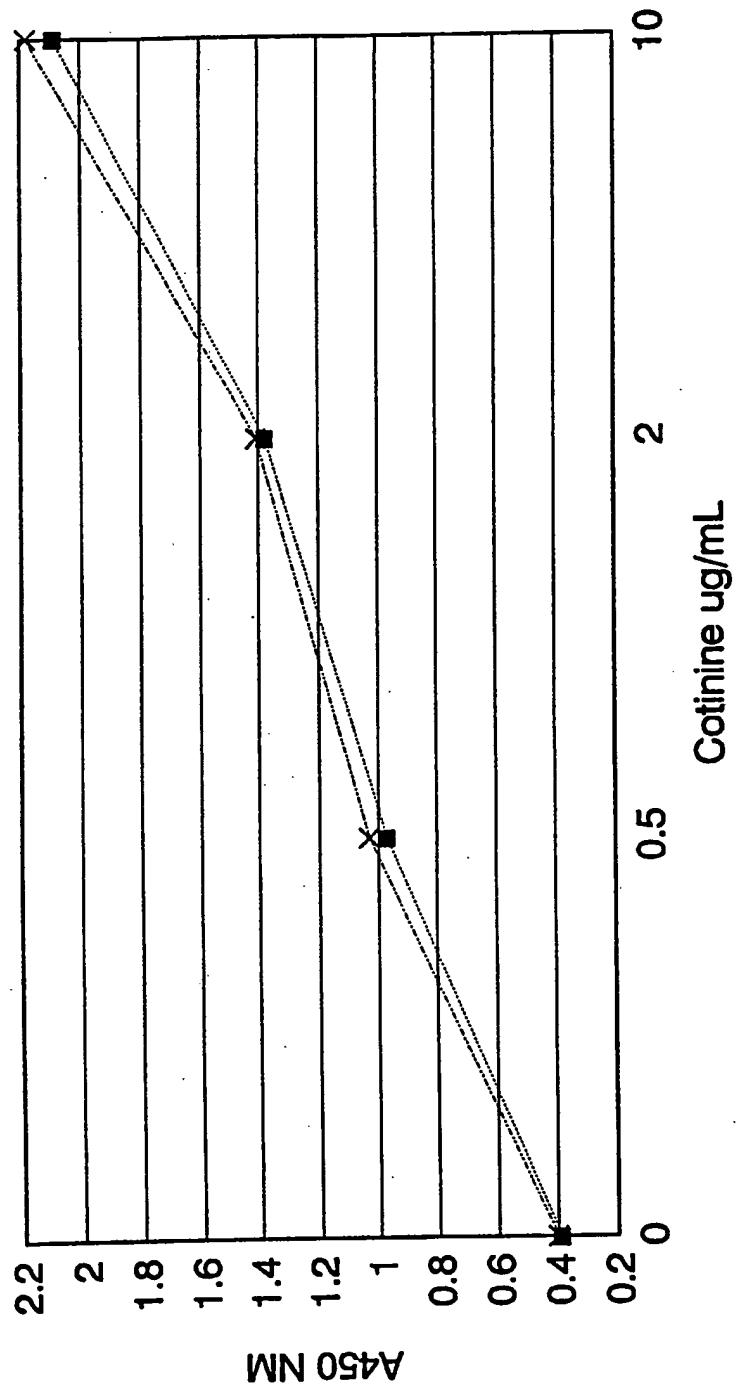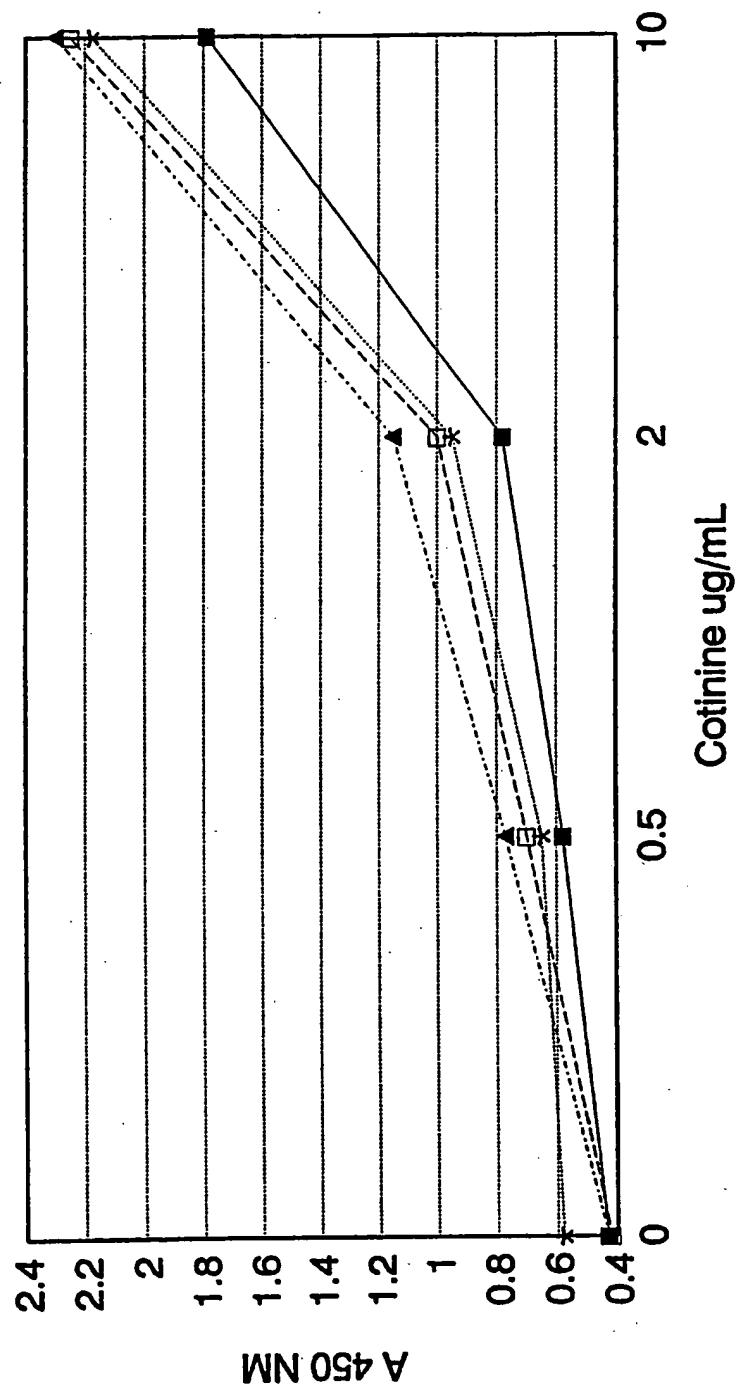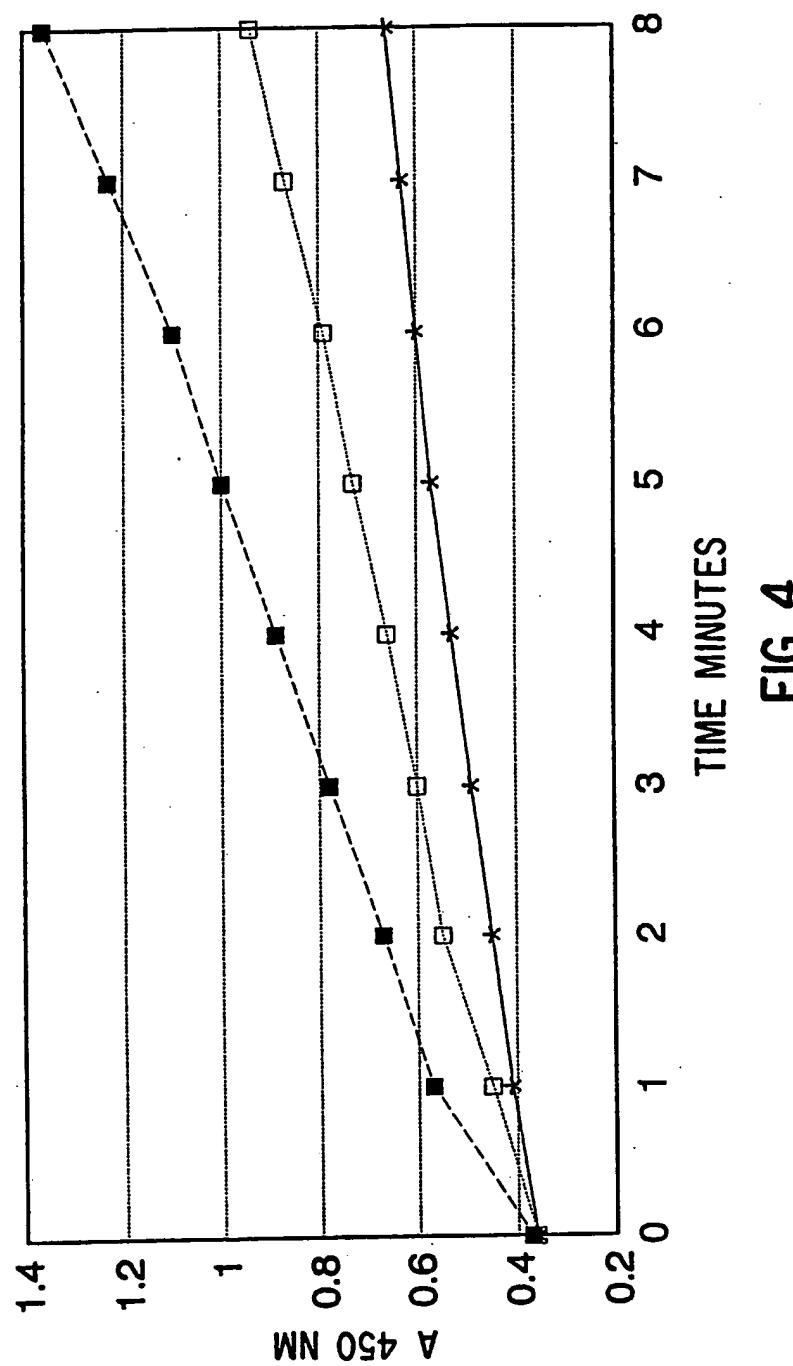
FIG. 1

2/14

FIG.2

FIG. 3

FIG. 4
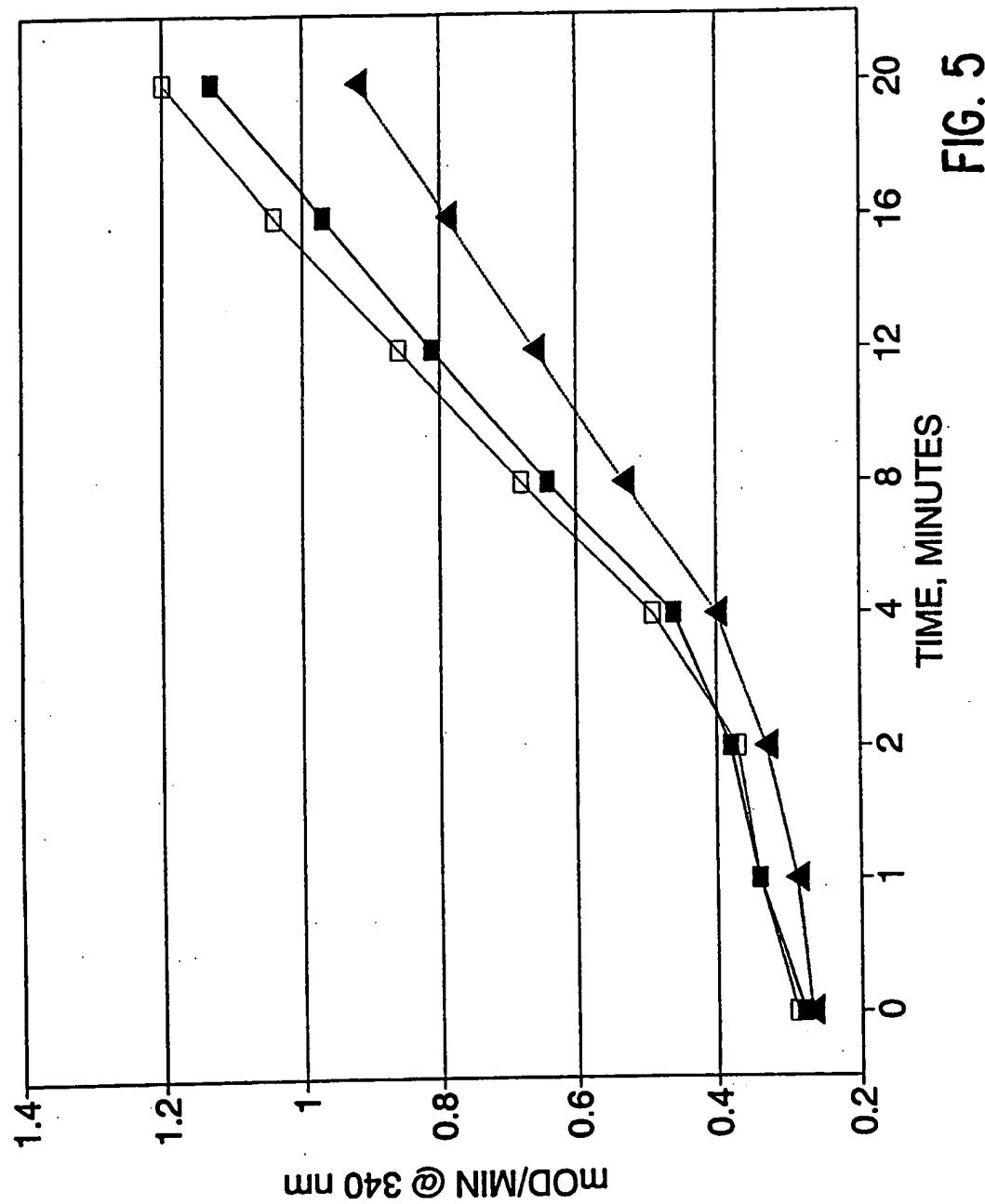
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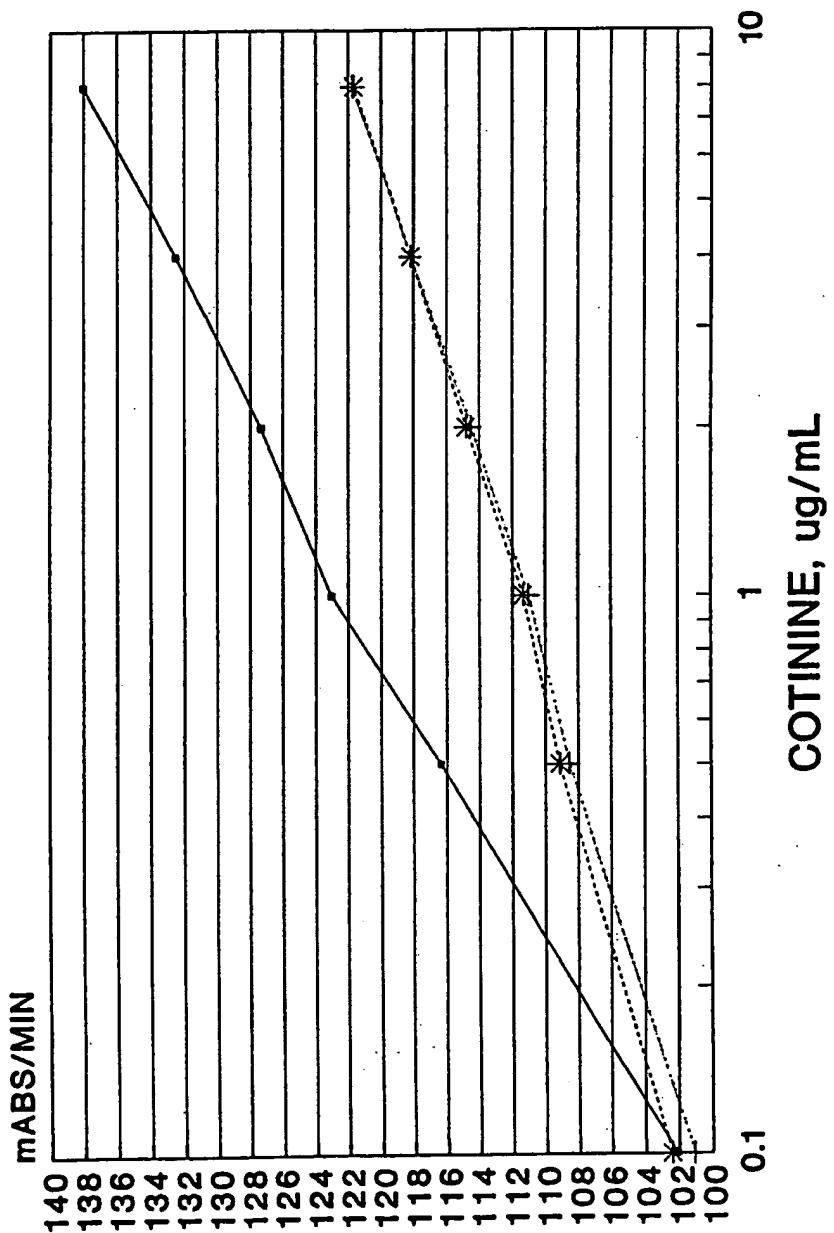
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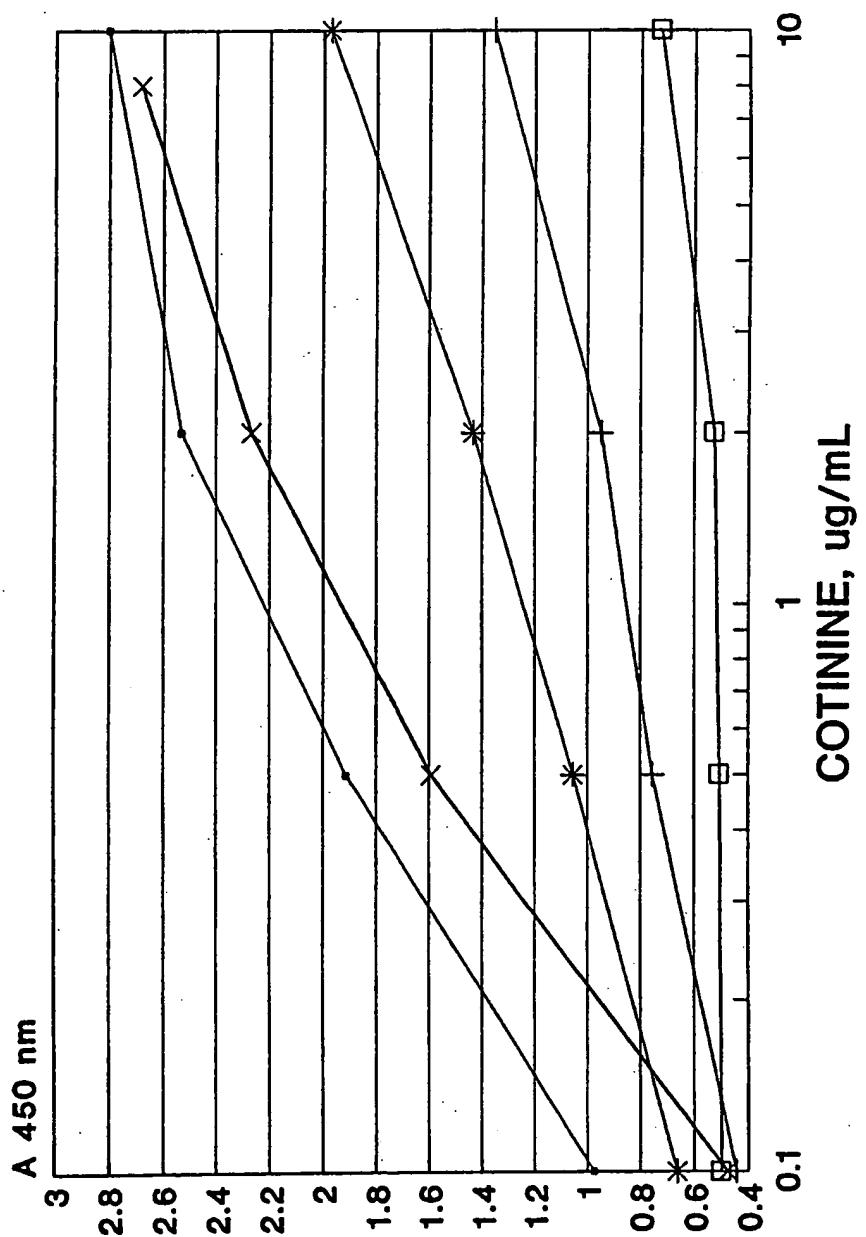
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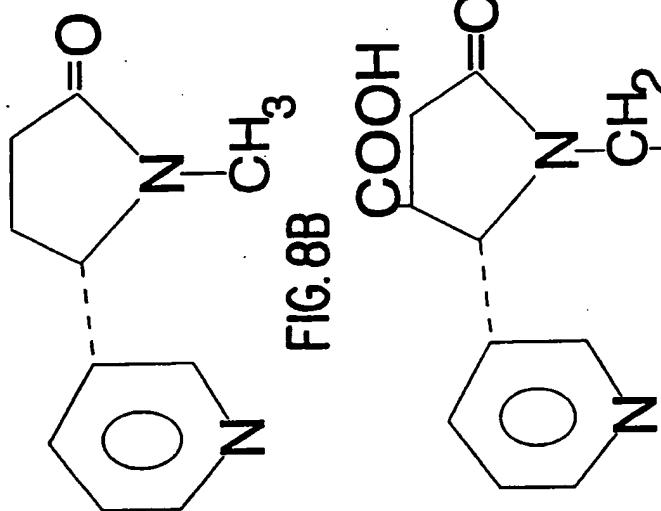
FIG.8B
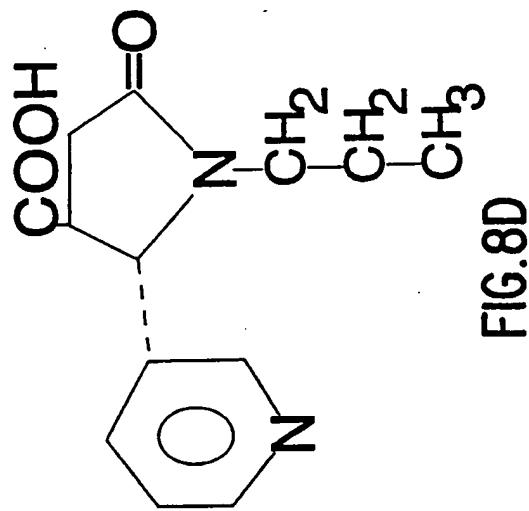
FIG.8D
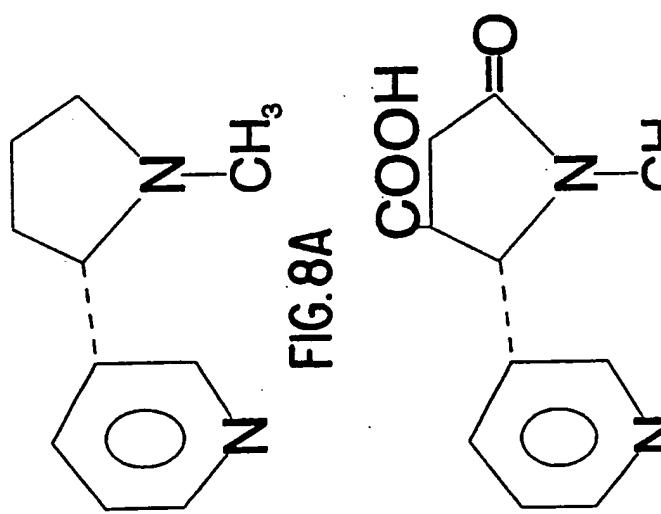
FIG.8A
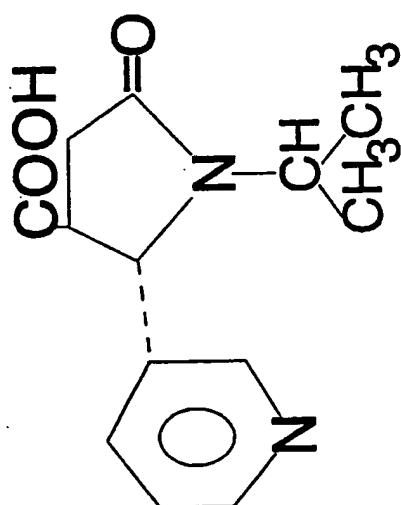
FIG.8C
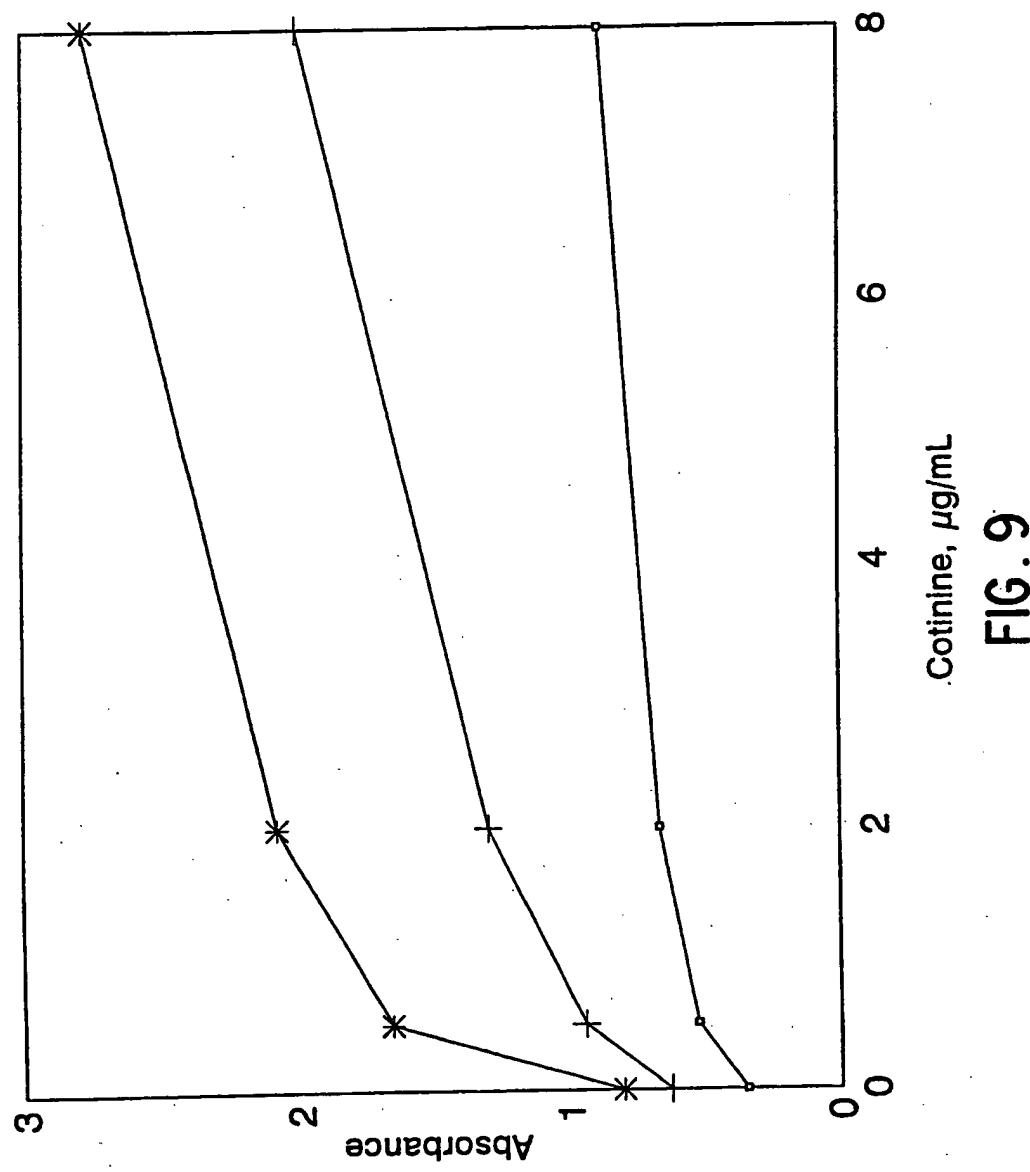
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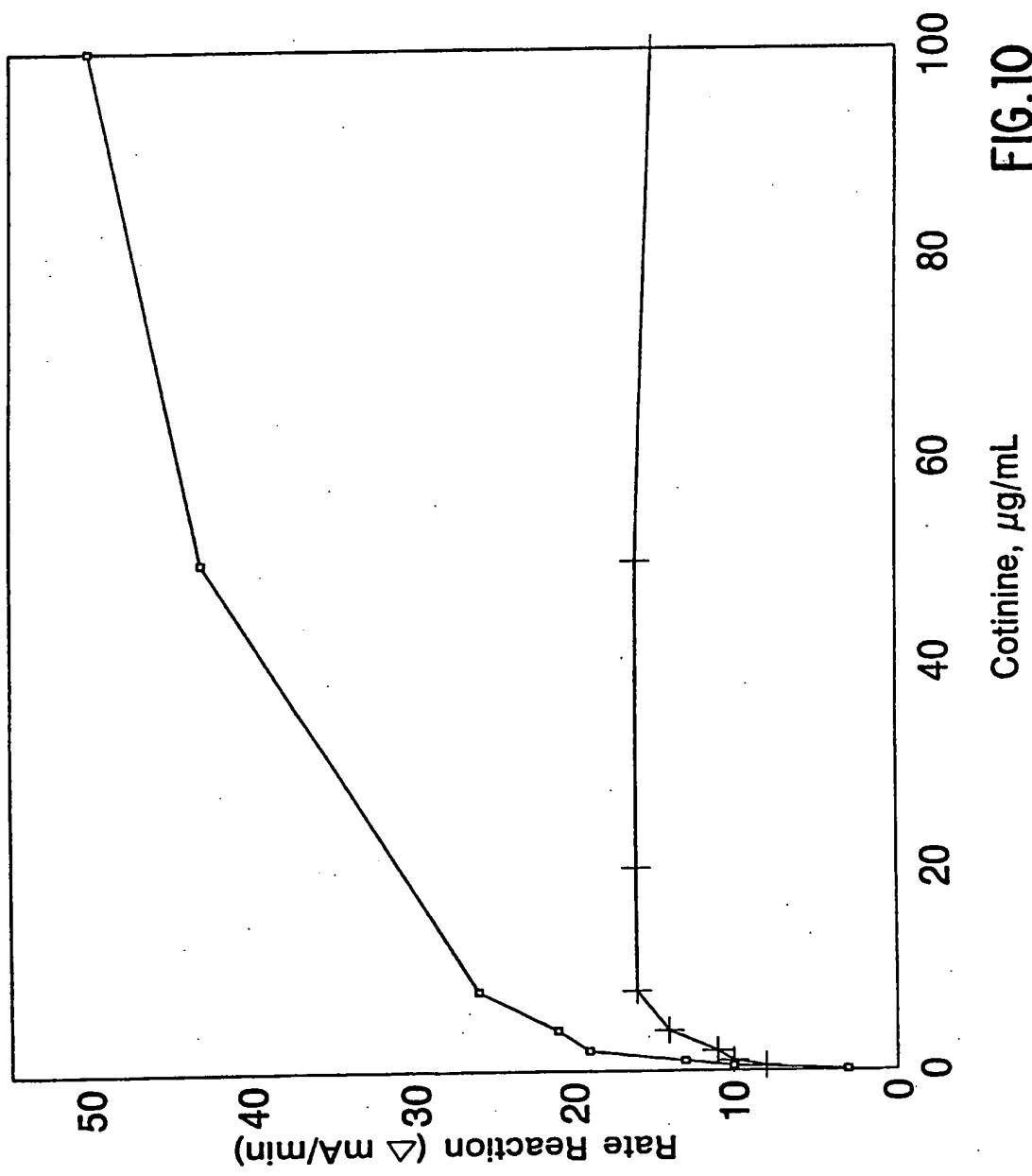
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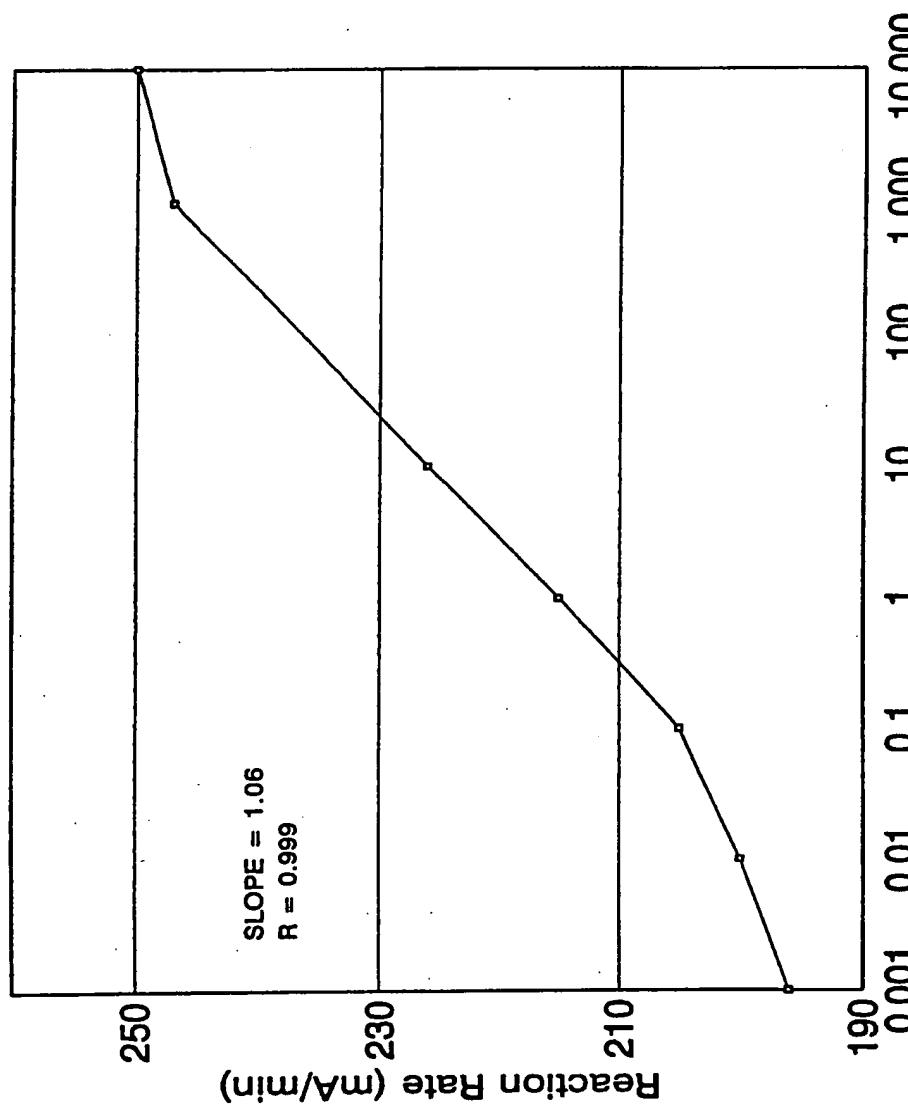
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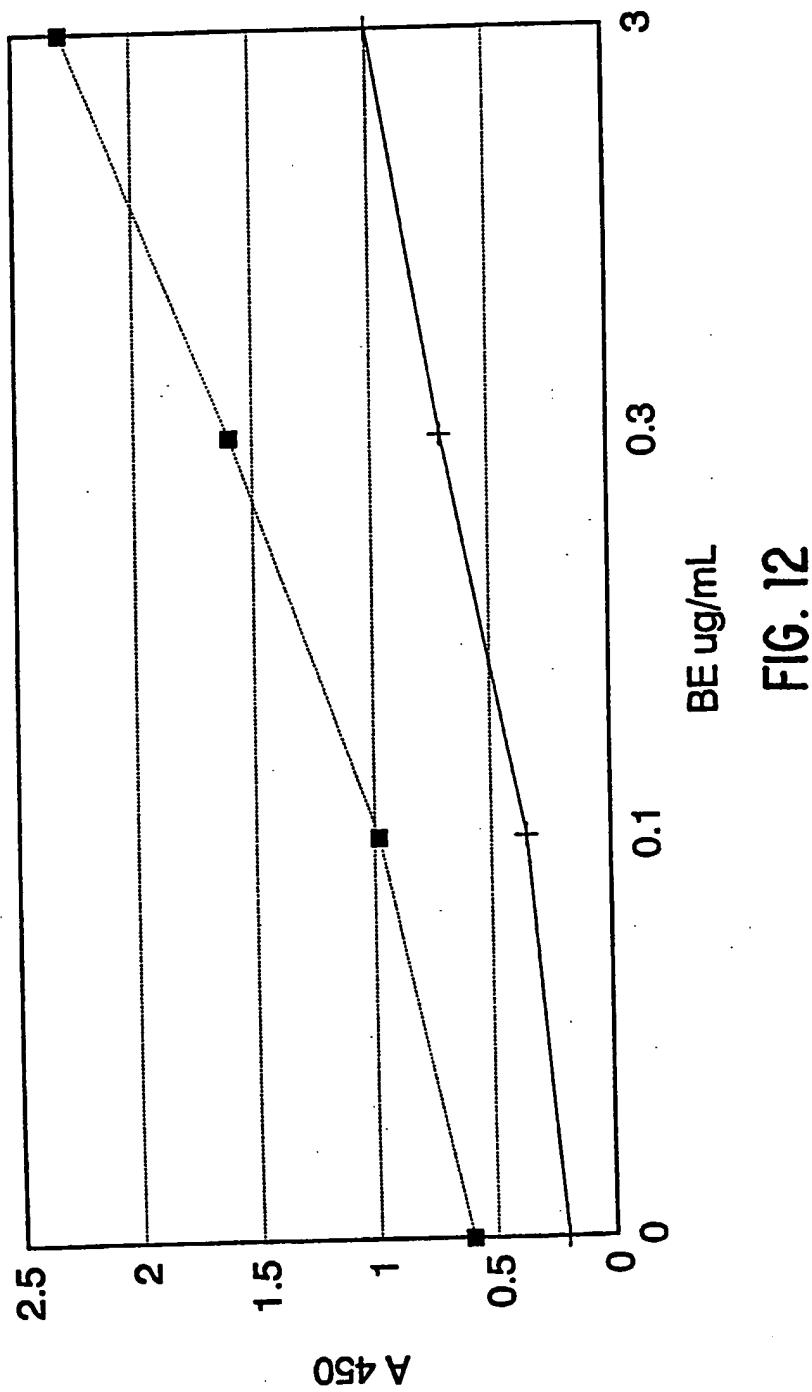
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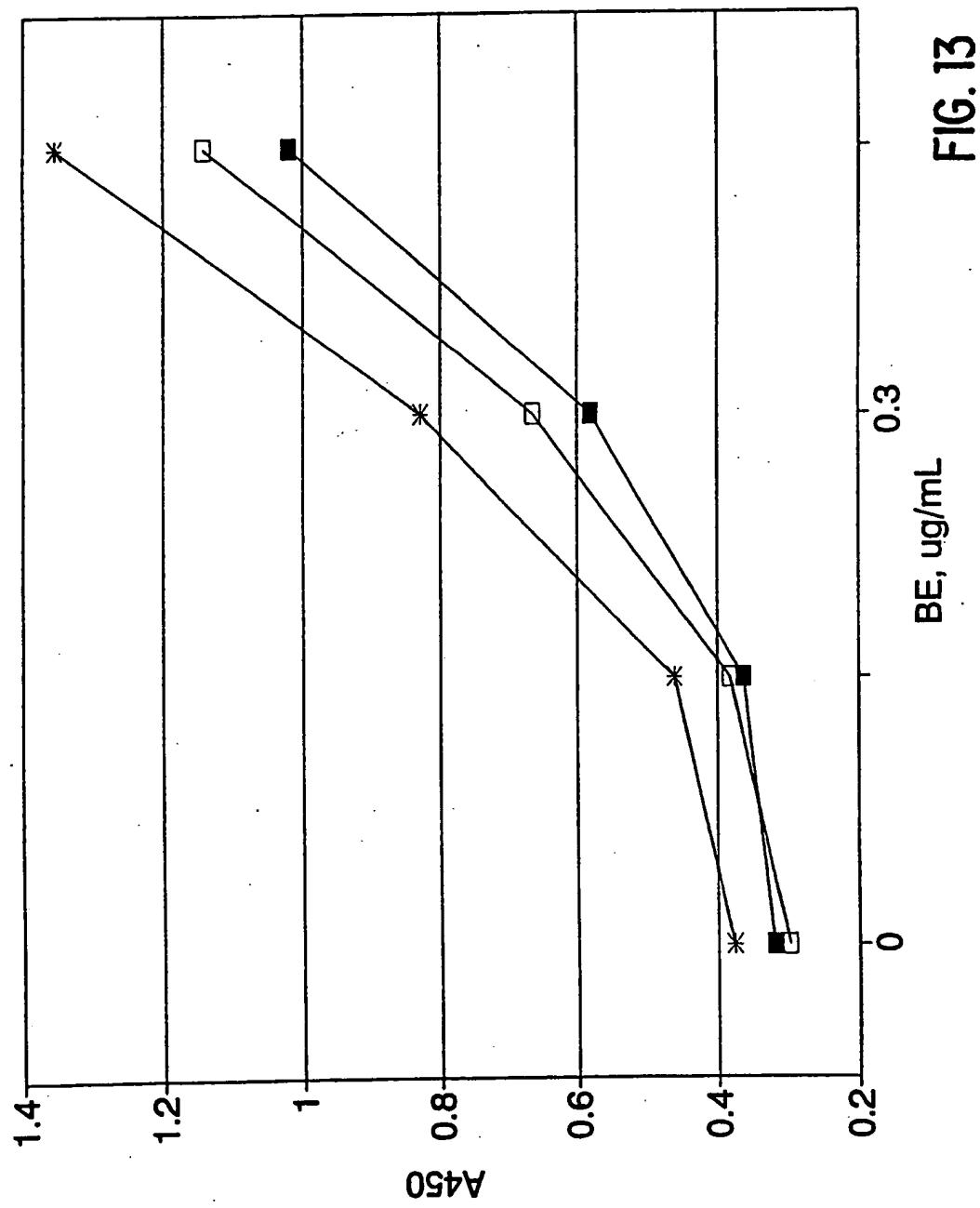
13/14

FIG. 13
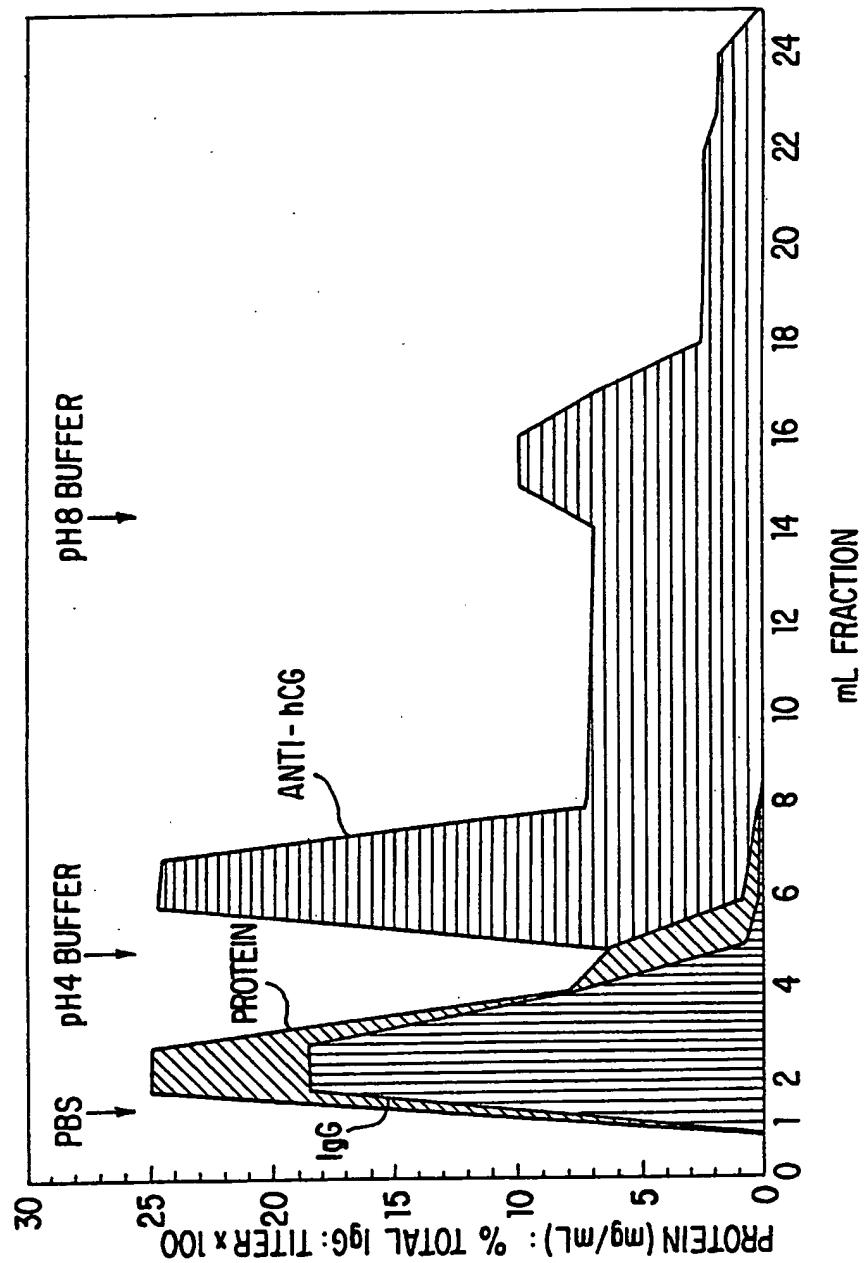
FIG. 14

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/53
US CL :435/7.9

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.9, 7.2, 7.93, 975; 436/518, 534, 541, 547, 804, 815, 816

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Online, Registry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 5,028,535 (Buechler et al) 02 July 1991, Abstract, col. 7, lines 12-17, col. 9, lines 59-63, col. 10, lines 13-21 and 36-40, paragraph bridging columns 10-11, and col. 22, lines 4-25.	1-6,8-11,13-14,16-19 7, 12, 15
Y	US, A, 4,115,535 (Giaver) 19 September 1978, abstract and col. 4, lines 4-8.	12
X Y	EP, A, 0,383,313 (Sato et al) 22 August 1990, page 4, lines 43-55, page 5, lines 56-57, page 7, lines 27-32, page 15, lines 42-56.	1-4,6,8-11, 14,16-19 5,7,12-13, 15
X Y	US, A, 4,383,031 (Boguslaski et al) 10 May 1983, col. 6, lines 34-64 and col. 5, lines 34-39.	1-4,6,8-11,14,16-19 5,7,12-13,15
Y	US, A, 3,917,582 (Soffer et al) 04 November 1975, Abstract, col. 1, lines 8-15.	5,7,13,15
X Y	Journal of Pharmaceutical Sciences, Volume 59, No. 3, issued March 1970, W. Hankins et al., "Pyrrolidine-Sustituted Nicotine Analogs: Synthesis and Pharmacology", pages 342-344.	20,21 22-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	
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"O"	document referring to an oral disclosure, use, exhibition or other means	
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Date of the actual completion of the international search	Date of mailing of the international search report
08 September 1992	**1 8 SEP 1992**
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SUSAN C. WOLSKI
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